HOST PLANT USE BY INSECT HERBIVORES MEDIATED BY MICROORGANISMS

A Dissertation

by

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ABSTRACT

Geographic isolation plays a major role in population divergence, but host-plants can also generate selective forces that drive genetic differentiation. Host-plant associations of insects can also be mediated by their associations with microorganisms, some of which are linked to pest status on crops. However, the ecological and evolutionary roles these microorganisms play in plant-insect interactions are not fully understood, neither are their implications for pest management. My dissertation applied an interdisciplinary approach to explore the potential role of microorganisms in plant-insect interactions. Two important agricultural pests, cotton fleahopper (CFH) (*Pseudatomoscelis seriatus*), a cotton pest, and sugarcane aphid (*Melanaphis sacchari*), an invasive pest of sorghum, were used as model organisms in my studies.

Using amplified fragment length polymorphisms (AFLPs) I sampled CFH from 12 host-plants. I found that CFH genotypes are distinct on only 2 out of 12 uncultivated plant species. My results suggest that several uncultivated hosts likely constitute a source of CFH for cotton. To determine whether bacterial symbionts correlated with genetic variation in CFH host-association, I compared bacterial communities within CFH using 454 pyrosequencing of the 16S rRNA gene. There was no correlation between the two.

Although CFH is widely distributed across cotton-growing regions in the U.S., it is considered a major cotton pest only in certain regions while in others it is rarely a pest. I compared bacterial communities within CFH using 16S rRNA pyrosequencing to test the potential for bacterial symbionts to influence variation in CFH pest status. Results from this

bacterial survey failed to find any correlation between variation in pest status of CFH and bacterial community composition.

I also explored the roles of potentially beneficial symbiotic fungi (i.e., fungal endophytes) in improving plant development and providing resistance against herbivory. Soaking sorghum seeds in liquid suspensions of two fungal endophytes increased seedling height and fresh biomass. Additionally, I detected that endophyte treatment significantly affected the reproduction and behavior of sugarcane aphids, but performance was conditional on the identity of the endophyte and the part of the plant where aphids were located. Overall, findings from my studies improve our understanding of the ecological and evolutionary implications of plant-microbe-insect interactions.

DEDICATION

To my mother, Margaret Antwi, I would not have accomplished this if it hadn't been for your support and encouragement. To my brothers, Alfred Antwi and Richard Owusu Banahene and my sister, Christina Tawiah, for their unfailing encouragement and love.

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CHAPTER I

INTRODUCTION

Insect herbivores exploit several host-plant species for food as well as use them for mating and shelter. After spending multiple generations on specific host-plant species, insect herbivores may adapt or specialize on those host-plants (Dickey & Medina 2011, 2012; Feder *et al.* 1993; Nosil & Crespi 2006). Such ecological divergence may result in adaptive traits responsible for reproductive isolation leading to the formation of genetically distinct host-associated lineages or host races. This phenomenon is commonly referred to as host-associated genetic differentiation (HAD). Existence of HAD may affect our ability to control agricultural pests in agro-ecosystems (Medina 2012). If pest populations are genetically distinct they could also differ in traits relevant to pest control and may require different pest control strategies. For example, knowledge of the existence of genetic structure in a pest population may improve the design of strategies that delay insecticide resistance, including resistance to transgenic crops (Carriere *et al.* 2010; Tabashnik *et al.* 2008).

Many insect herbivores traditionally considered to be generalist species are actually composed of genetically distinct and reproductively isolated populations (Fox & Morrow 1981). However much of what we know about HAD involves insect species with narrow host ranges (i.e., specialists) that spend part or all their life cycle inside their hosts, and/or reproduce asexually (e.g., parthenogenetically). When sexually reproducing polyphagous insects have been tested for HAD only a handful of their host-plant species were tested. Currently we do not know how widespread HAD is in many polyphagous sexually reproducing insects, and much less in insect pests within many agroecosystems.

One notable aspect of plant-insect interactions is that sometimes each player (i.e., plants and insects) establishes symbiotic associations with microorganisms that provide insects with net fitness benefits when under variable biotic and abiotic conditions (Gundel *et al.* 2012; Oliver *et al.* 2010). In fact, several experimental studies confirm that insect-microbe and plant-microbe symbiotic associations are ubiquitous phenomena in nature (Douglas 2009; Vega *et al.* 2009). For example, microbial symbionts provide insect herbivores with essential nutrients that are lacking in food plants, as well as protect insects against natural enemies (Douglas 1998; Oliver *et al.* 2010). Microbial symbionts of plants fulfill similar roles through uptake of nutrients and protection against insect herbivores and pathogens (Berg 2009; Elena *et al.* 2001). Thus, studies that take the roles of microbes in plant-insect interactions into account are needed for a complete understanding of this interaction.

For insect herbivores, symbiotic associations with bacteria have long been known to facilitate colonization of new feeding niches and allow insects to specialize on a broad range of diets (Douglas 2009). There is evidence that specialization on different host-plant species through associations with bacterial symbionts results in population divergence of insect herbivores (Ferrari & Vavre 2011; Medina *et al.* 2011). Thus, intraspecific variation encoded within the nuclear or organellar genomes of insects is not the only driving force of HAD; associated bacterial symbionts are also important components in the evolution of plant-insect interactions (Feldhaar 2011; Ferrari & Vavre 2011). However, much of our knowledge about the roles that bacterial symbionts can play in HAD comes from studies involving a handful of insect species, including several belonging to Aphididae (Brady & White 2013; Ferrari *et al.* 2007; Tsuchida *et al.* 2004) or insects belonging to Phyloxeridae (Medina *et al.* 2011). Additionally, studies investigating the effects of symbionts on HAD at the microbiome level

are rare, and given that several insect herbivores are not just infected with a single bacterial symbiont but with multiple bacteria, it is important to consider the influence of an insect's microbiome on HAD

Interestingly, the ability of bacterial symbionts to enable insect herbivores to colonize new feeding niches has also been implicated in facilitating the invasiveness of insects. For example, certain populations of whiteflies (*Bemisa tabaci*), plastaspid bean bugs (*Megacopta punctatissima*) and kudzu bugs (*Megacopta cribraria*) are crop pests and their pest status have been linked to their associations with symbiotic bacteria (Brown *et al.* 2013; Gueguen *et al.* 2010; Hosokawa *et al.* 2007). However, despite these prominent examples, studies linking bacterial symbionts to pest status are surprisingly rare to date given that a large number of pest species also harbor bacterial symbionts (Chu *et al.* 2013; Hirose *et al.* 2006; Prado *et al.* 2009; Toju *et al.* 2011).

Bacterial symbionts may allow insect herbivores to successfully feed on particular plants. However, symbiotic interactions between plants and microorganisms also occur. For example, endophytes are microorganisms that can colonize a plant without causing visible disease symptoms. Among these, certain fungal endophytes have been shown to confer resistance to insect herbivores as well as improve plant development (Vega *et al.* 2009). In recent years, there has been an increasing interest in the use of horizontally transmitted fungal endophytes in integrated pest management (IPM) as a means to reduce insecticide applications on row crops. However, endophyte-mediated defense against insect herbivores is a variable phenomenon conditional on the type of endophyte or the insect species. Very few studies have tested this idea, especially in field crop insects. A more complete understanding

of the relative response of insects feeding on endophyte-treated plants will help promote the effective use of fungal endophytes as part of successful IPM strategies.

The overall goal of my dissertation was to provide basic information that will broaden our understanding of the evolutionary and ecological consequences of plant-insect interactions and the role that microbes play in this interaction. I used two model pest-plant systems, the cotton fleahopper (CFH), *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae) as a major pest of cotton, Gossy*pium hirsutum* L., and the sugarcane aphid,*Melanaphis sacchari* (Zehntner) (Hemiptera: Aphididae) as a new invasive pest of sorghum,*Sorghum bicolor* L. (Moench). CFH is an economically important pest of cotton in Texas, Oklahoma and Arkansas. Cotton is most vulnerable to CFH attack during the first three weeks of early flower bud (referred as "squares") development (Sansone *et al.* 2009). In Texas alone, average CFH-induced yield loss estimates vary, reaching up to 6% with an estimated lost income ranging from ~$3 million to ~$7 million (Williams 2010). The sugarcane aphid, on the other hand, is an invasive pest on sorghum. It was first noticed as a pest on sorghum in parts of Louisiana and east Texas in 2013 (Way 2014). It has since spread throughout the south-central U.S. (Armstrong *et al.* 2015) where it can have negative effects on sorghum development, yield and harvest.

My specific objectives and hypotheses were:

1. To test the frequency of HAD in a highly polyphagous sexually reproducing insect, cotton fleahopper (CFH) (chapter II). CFH provides a good model to test for HAD because it feeds on at least 160 host-plant species belonging to 35 different families of both managed crops and unmanaged wild plants. Additionally, a recent study tested HAD in CFH on three of its host-plants and detected one incidence of

HAD (Barman *et al.* 2012). Given that CFH is highly polyphagous, I hypothesized that HAD is likely to occur on other host-plant species as well.

2. To test the role of bacterial communities in mediating HAD in host-associated CFH populations (chapter III). Based on previous knowledge of the existence of HAD in CFH and the potential role for bacterial symbionts to influence genetic variation of insects, I hypothesized that HAD in CFH would correlate with variation in the insect's microbiome.

3. To determine whether bacterial communities influence the pest status of CFH in the cotton-growing regions of the U.S. (chapter IV). Given that bacterial symbionts have been linked to the emergence of pest status of sucking insects, I hypothesized that variation in pest status of CFH would correlate with variation in microbiome of the insect.

4. To determine the effect of two fungal endophytes (*Beauveria bassiana* and *Purpureocilium lilacinum*) on the growth of sorghum and their impact on the behavior and performance of sugarcane aphids (chapter V). I hypothesized that these fungal endophytes would not change the growth of sorghum. This hypothesis is based on mixed results from previous studies using *B. bassiana* and the fact that *P. lilacinum* has never been tested on sorghum. However, I expected endophyte to have a negative effect on aphid performance and behavior based on observations from previous studies.

A successful implementation of any IPM strategy relies on both economically and ecologically sound pest control methods that are sustainable and environmentally friendly. However, implementation of any such method requires a complete understanding of host-

plant use by insects. In recent years, where microbial control of insect pests is becoming more and more common in agricultural practices (Tabashnik *et al.* 2008; Vega *et al.* 2009), a good understanding of their functional role in plant-insect interactions is needed. In my dissertation, I employed an interdisciplinary approach using both evolutionary and applied ecology to explore the potential role of microorganisms in modulating plant-insect interactions. Information gathered from this dissertation will help improve our understanding of plant-insect interactions in multiple ways. First, it will provide baseline information about the existence (or a lack thereof) of intra-specific genetic variation of CFH associated with a wide range of host-plants, including cotton and how it may impact CFH control on cotton fields. Secondly, in addition to the evolutionary implications a correlation between genetic distinctness and microbiome data from my studies will have implications for the insect's competence as a vector to transmit pathogens into cotton. This is because successful development, survival and transmission of pathogens from a vector to a host depend on genetic compatibility of a vector-pathogen association. Third, if the microbiome of CFH has any influence on the feeding ecology of CFH, variation in microbiome composition may help explain the geographic variation in the pest status of the CFH. Finally, my dissertation will contribute to our understanding of plant and insect responses to artificial inoculation of plants with fungal endophytes. Information gathered from this study will not only help inform alternative methods to control the sugarcane aphid, but will also provide insight into the differential responses of insects feeding on endophyte-treated plants.

CHAPTER II

HOST-ASSOCIATED DIFFERENTIATION IN A HIGHLY POLYPHAGOUS, SEXUALLY REPRODUCING INSECT HERBIVORE[*]

Synopsis

Insect herbivores may undergo genetic divergence on their host plants through host-associated differentiation (HAD). Much of what we know about HAD involves insect species with narrow host ranges (i.e., specialists) that spend part or all their life cycle inside their hosts, and/or reproduce asexually (e.g., parthenogenetic insects), all of which are thought to facilitate HAD. However, sexually reproducing polyphagous insects can also exhibit HAD. Few sexually reproducing insects have been tested for HAD and when they have, insects from only a handful of potential host plant populations have been tested, making it difficult to predict how common HAD is when one considers the entire species' host-range. This question is particularly relevant when considering insect pests, since host-associated populations may differ in traits relevant to their control. Here, we tested for HAD in a cotton (*Gossypium hirsutum*) pest, the cotton fleahopper (CFH) (*Pseudatomoscelis seriatus*), a sexually reproducing, highly polyphagous hemipteran insect. A previous study detected one incidence of HAD among three of its host plants. We used AFLP markers to assess HAD in CFH collected from an expanded array of 13 host plant species belonging to 7 families. Overall, 4 genetically distinct populations were found. One genetically distinct genotype was exclusively associated with 1 of the host-plant species while the other 3 were observed across

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more than one host-plant species. The relatively low degree of HAD in CFH compared to the pea aphid, another hemipteran insect, stresses the likely importance of sexual recombination as a factor increasing the likelihood of HAD.

Introduction

Host plants play an important role in the diversification of insect populations (Ehrlich & Raven 1964). While associated with different host plant species, insect populations can experience different selection pressures that may create ecological barriers to gene flow (Feder *et al.* 1993; Nosil & Crespi 2006; Pashley 1986). Divergent selection on different host-plant species may result in adaptive traits responsible for reproductive isolation among host-associated subpopulations. If reproductive isolation is maintained, this process may end up in the formation of genetically distinct host-associated lineages or host races (Bernays 1991; Carroll & Boyd 1992; Diehl & Bush 1984; Dres & Mallet 2002; Pappers *et al.* 2001). This phenomenon is commonly referred to as host-associated differentiation (HAD) (Abrahamson *et al.* 2001; Bush 1969).

In recent years, there has been a growing interest in HAD and several studies have sought to investigate the phenomenon in a variety of insect species including specialist (Althoff *et al.* 2007; Funk *et al.* 2002; Heard 2012; Hernadez-Vera *et al.* 2010; Medina *et al.* 2012) and generalist insects (Barman *et al.* 2012; Funk *et al.* 2002; Sword *et al.* 2005). Perhaps, some of the best studied cases of insect HAD are those involving apple maggot flies (*Rhagoletis pomonella* Walsh) on apples and hawthorns (Bush 1969; Feder *et al.* 1993; Forbes *et al.* 2010), species associated with goldenrods (Abrahamson *et al.* 2001; Eubanks *et al.* 2003; Stireman *et al.* 2005), pea aphids (*Acyrthosiphon pisum* Harris) associated with

plants in the Fabaceae family (Frantz *et al.* 2006; Peccoud *et al.* 2009; Via 1999), and stick insects (*Timema cristinae)* on redheart and chamise (Nosil et al., 2002, Soria-Carrasco et al., 2014). In all these insect species, genetically distinct lineages have been found on different host-plant species. In fact, the remarkable diversity of insects we see today could be the result of HAD (Abrahamson *et al.* 2001; Dres & Mallet 2002; Farrell 1998), making the study of HAD an important component in our understanding of the role of host-plant species in ecological speciation.

Level of intimacy with their hosts (i.e., whether an insect lives/feeds within plant tissues vs. externally) and the type of reproduction (i.e., sexual or asexual) are factors thought to influence the propensity of insects to exhibit HAD (Medina 2012). Much of what we know about HAD involves insect species with narrow host ranges (i.e., specialists) that spend part or all their life cycle inside their hosts, and/or reproduce asexually (e.g., parthenogenetically) (Brunner *et al.* 2004; Cook *et al.* 2012; Darwell *et al.* 2014; Dickey & Medina 2010, 2012; Marques *et al.* 2014; Pashley 1986; Van Zandt & Mopper 1998). Pea aphids, for example, are parthenogenetic Fabaceae specialists that are composed of genetically distinct host-associated lineages on clover and alfalfa (Via 1999). Even though pea aphids are associated with multiple plant species (Ferrari *et al.* 2006; Frantz *et al.* 2006; Simon *et al.* 2003; Via 1999), it was not until Peccoud *et al.* (2009), sampled insects from an extensive number of different host-plant populations that HAD in pea aphids was found to be more extensive than previously thought. This raises the question of whether HAD is really uncommon in sexually reproducing generalists or perhaps has simply been overlooked due to limited sampling.

Evidence of HAD in sexually reproducing generalist species is accumulating. For example, grasshoppers and green mirids are polyphagous, feeding on multiple hosts from

different families, yet they exhibit HAD (Apple *et al.* 2010; Hereward *et al.* 2013; Sword & Dopman 1999; Sword *et al.* 2005). For agriculturally important pests, genetically distinct lineages on different host plants may differ in their susceptibility to certain pest control methods. Thus, knowing which pest species show HAD is important. For example, conservation biological control may not work in a particular crop if natural enemies co-evolve with their insect hosts on one host plant species and become reproductively isolated on alternative host-plants (Eubanks *et al.* 2003; Forbes *et al.* 2010). Similarly, the use of alternative host-plant species as refuges in transgenic crop plantings, may not work if host associated populations of polyphagous pests are reproductively isolated when on different host-plant species (Calcagno *et al.* 2007). Although some sexually reproducing generalist pests (e.g., fall armyworm, browntail moth, green mirid) have been shown to exhibit HAD (Hereward *et al.* 2013; Marques *et al.* 2014; Pashley 1986) we currently do not know how widespread HAD is across the agroecosystems in which these pest species exist.

The cotton fleahopper (CFH), *Pseudatomoscelis seriatus* Rueter, (Hemiptera: Miridae) offers a good model to test HAD in a sexually reproducing generalist insect pest in a highly managed monoculture. CFH feeds on at least 160 host-plant species belonging to 35 different families of both managed crops and unmanaged wild plants (Esquivel & Esquivel 2009; Snodgrass *et al.* 1984). It feeds using its piercing-sucking mouthparts on anthers and young flower buds of developing plants. As an agriculturally important crop, cotton (*Gossypium hirsutum*) is most vulnerable to CFH attack during the first three weeks of early flower bud (referred as "squares") development (Sansone *et al.* 2009). Recently, Barman *et al.* (2012) tested for HAD in CFH when associated with three of its most abundant host-plants in Texas, USA: horsemint, *Monarda punctata* L., woolly croton, *Croton capitatus* and

cotton. CFH on horsemint showed strong HAD in areas where annual precipitation was below 26 inches. Given that CFH is highly polyphagous, we predicted that HAD would be likely to occur on other host-plant species as well. To test this hypothesis, we used AFLP and Bayesian analyses to test for HAD among CFH collected from 13 different host-plant species belonging to 7 plant families.

Materials and Methods

Cotton Fleahopper Sampling and Host Plant Identification

I sampled CFH from 13 host plant species (belonging to 7 families), one of which is an annual crop (cotton) and 12 perennial plants (Table 1). Plant families sampled included: Asteraceae, Euphorbiaceae, Lamiaceae, Malvaceae, Onagraceae, Solanaceae, and Verbeneceae. CFH were collected from 14 locations in Texas, spanning 12 counties distributed across multiple ecological regions from the Piney Woods in the east to the High Plains in the west (Figure 1).

In addition to collecting CFH individuals, I collected plant samples from which the insects were collected as voucher specimens. Plants were individually pressed using standard plant press protocols (Queensland-Herbarium 2013). Plants were identified to species by Dr. Dale Kruse (S. M. Tracy Herbarium, Department of Rangeland Ecology and Management, TAMU, College Station, Texas).

Table 1. Host-plants and sampling locations of CFH in Texas. Letters in parenthesis are abbreviations of common names of host-plants.

Location (County)	Species name	Common name	Family name
(Travis)			
Leander	*Hymenopappus scabiosaeus* L'Hér.	Old plainsman (OP)	Asteraceae
Austin	*Malvella lepidota* Gray	Scurvy mallow (SM)	Malvaceae
(Brazos)			
College Station	*Ambrosia psilostachya* DC.	Western ragweed (WR)	Asteraceae
	Oenothera speciose Nutt.	Evening primrose (EP)	Onagraceae
	Gaura parviflora Douglas	Velvet-leaf beeblossom (VB)	Onagraceae
	Gossypium hirsutum L.	Cotton (CT)	Malvaceae
	Monarda punctate L.	Horsemint (HM)	Lamiaceae
	Solanum elaegnifolium Cav.	Silverleaf nightshade (SN)	Solanaceae
(Nueces)			
Corpus Christi	*Oenothera speciose* Nutt.	Evening primrose (EP)	Onagraceae
	Gossypium hirsutum L.	Cotton (CT)	Malvaceae
	Monarda punctate L.	Horsemint (HM)	Lamiaceae
	Solanum elaegnifolium Cav.	Silverleaf nightshade (SN)	Solanaceae
(Lubbock)			
Lubbock	*Gossypium hirsutum* L.	Cotton (CT)	Malvaceae
	Monarda punctate L.	Horsemint (HM)	Lamiaceae
	Solanum elaegnifolium Cav.	Silverleaf nightshade (SN)	Solanaceae
(Burnet)			
Burnet	*Malvella lepidota* Gray	Scurvy mallow (SM)	Malvaceae
	Marrubium vulgare L.	Common horehound (CH)	Lamiaceae
	Glandularia bipinnatifida Nutt.	Purple praire (PP)	Verbaneceae
	Hymenopappus scabiosaeus L'Hér.	Old plainsman (OP)[a]	Asteraceae
	Solanum elaegnifolium Cav.	Silverleaf nightshade (SN)	Solanaceae
(Comal)			
Fisher	*Croton monanthogynus* Michx.	Oneseed croton (OC)	Euphorbiaceae
(Guadalupe)			
Sequin	*Croton lindheimerianus* Scheele	Threeseed croton (TC)	Euphorbiaceae
(Henderson)			
La Rue	*Croton argyranthemus* Michx.	Silvercroton (SC)	Euphorbiaceae
(Real)			
Barksdale	*Marrubium vulgare* L.	Common horehound (CH)	Lamiaceae
(Tom Green)			
San Angelo	*Monarda punctate* L.	Horsemint (HM)	Lamiaceae
	Gossypium hirsutum L.	Cotton (CT)	Malvaceae
(Hildago)			
Weslaco	*Monarda punctate* L.	Horsemint (HM)	Lamiaceae
	Gossypium hirsutum L.	Cotton (CT)	Malvaceae

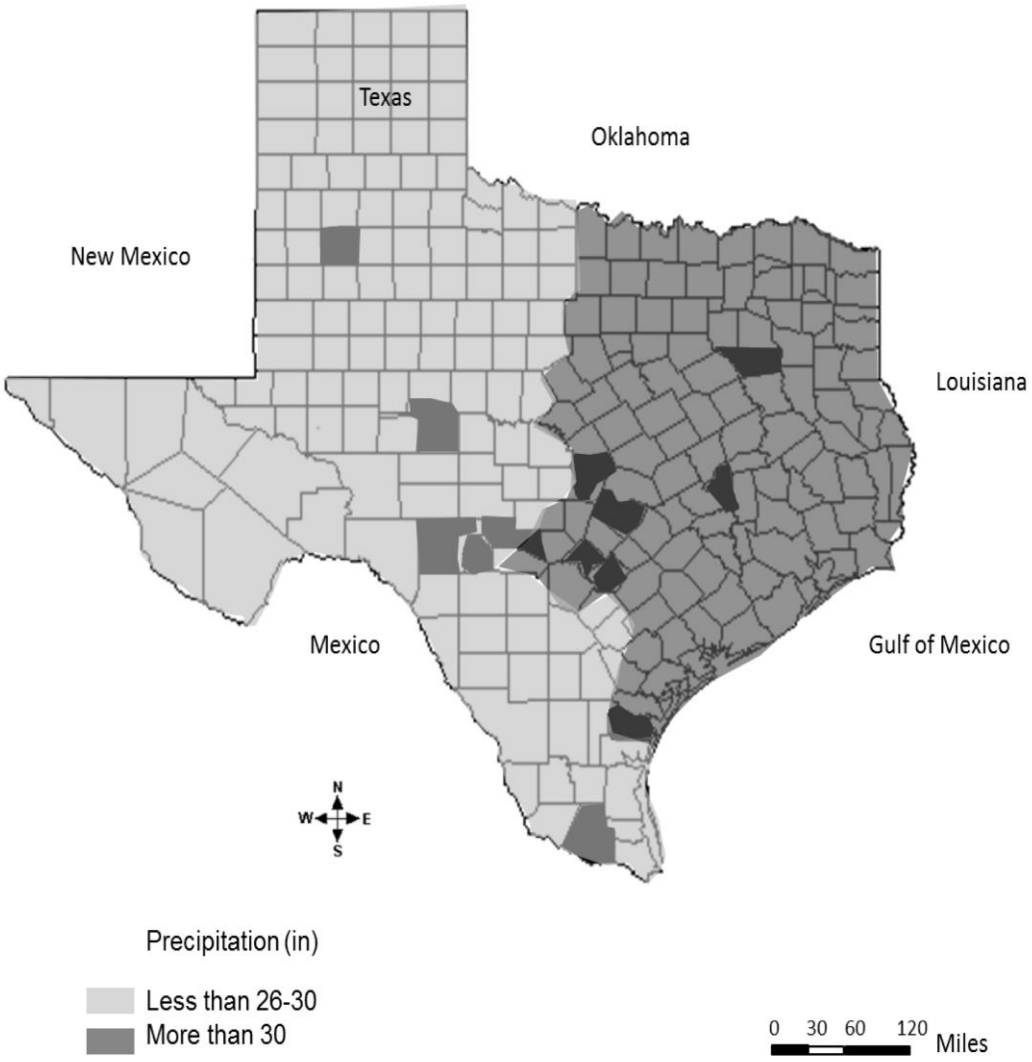

Figure 1. Map of Texas ecoregions indicating locations where CFH was sampled. Sampling locations are indicated in dark gray black. The entire state is divided into two regions with respect to annual precipitation as described by Barman et al., (2012)

Cotton fleahopper sampling took place during the spring and summer of 2013 and 2014 when herbaceous plants had green foliage, some of which were blooming at the time of sampling. On cotton, CFH sampling coincided with the development of flower buds ("squares") when CFH numbers were typically high. Using hand-held sweep nets and aspirators, insects were sampled from cotton fields, wild vegetation patches surrounding cotton fields, open fields within natural forest stands, and along roadsides and highways. I initially planned to sample only CFH nymphs from each host-plant, however, due to overall low nymph numbers on several of the host-plants sampled, I also included adults in this study. In all, a total of 240 individuals were analyzed, ranging from 8 to 20 individuals per host-plant species per location. Individuals were stored in 80% ethanol prior to DNA extraction.

DNA Extraction and AFLPs

Genomic DNA was extracted from whole insects using DNeasy® tissue extraction kit (QIAGEN Valencia, CA, USA) following the manufacturer's protocol and stored in AE buffer at -20^0C. DNA concentration and quality were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). On average, DNA concentration and quality from individual CFH extractions were 100 ng/µl and 2.00, respectively. Amplified fragment length polymorphism (AFLP) reactions were performed following the protocol of Vos *et al.* (1995) with minor modifications by Barman *et al.* (2012). Briefly, aliquot of DNA from individuals were randomly assigned to a 96-well plate, repeating one control individual three times on each plate to assess reproducibility. A negative control (blank) was included in every plate to assess potential cross-contamination.

A restriction digestion of 5.5µl DNA and 5.5 µl of master mix containing 0.03 µl T4 DNA ligase (New England Biolabs (NEB), Ipswich, MA, USA), 1.1 µl 10x T4 DNA ligase buffer, 1.1 µl 0.5M NaCl, 0.55 µl diluted BSA, 0.05 µl MseI (NEB), 0.05 µl EcoRI (NEB), 1 µl each MseI and EcoRI adapter pairs (Life Technologies), and 0.61 µl sterile distilled water was performed. Reactions were incubated overnight (approximately 16 hrs) after which they were diluted 17-fold, with 189 µl TE_{thin} buffer. This was followed by a 20 µl total volume pre-selective PCR reaction mix consisting of 4 µl diluted DNA, 15 µl AFLP core mix (Life Technologies), and 1 µl AFLP amplification primers (Life Technologies). Selective PCR amplifications were performed in a 21 µl volume of 15 µl platinum supermix (Life Technologies), 4 µl of a 19 fold diluted pre-amplification reaction product, and one primer combination consisting of 1 µl MseI-CAT (Life Technologies) and 1 µl EcoRI-ACT (Life Technologies). All PCR amplifications were carried out in an ABI GeneAmp thermocycler (Life Technologies) using protocols from Barman *et al.* (2012). Reactants were prepared in a laminar flow hood. DNA and PCR reagents were added using filter tips to minimize the risk of cross contamination. A 10.5 µl total volume consisting of 1 µl selective amplification PCR product, 9 µl HiDi formamide, and 0.5 µl ROX 400 size standard (Life Technologies) were used for electrophoretic analysis of selective PCR fragments. Samples were analyzed on an ABI 3730xl 96-capillary genetic analyzer (Applied Biosystems, Forest City, CA, USA).

Genetic Analysis

AFLP fragments were analyzed with the genetic software GeneMarker v.2.6.3 (Softgenetics). Only loci with fragment sizes within 50 to 400 bp, and florescent units of 100 or more were included in our analyses. Results from GeneMarker were converted into a

binary matrix of presence (1) or absence (0) for each locus. Loci with fewer than 5% markers than the average number of markers per loci were removed from the dataset. Fragment amplification failed in 30 individuals that were accordingly removed from the dataset. To ascertain whether the number of individuals and the number of markers used in the study were sufficient to accurately predict genetic structure of CFH, we used the SESim statistic (Medina *et al.* 2006). The SESim statistic uses a resampling technique to sub-sample the standard error of mean similarity indexes of individuals and loci (Median *et al.* 2006). A SESim value lower than 0.05 indicates that the number of loci and individuals in a dataset are sufficient and that additional markers or individuals may not alter the population clustering pattern produced by the sampled area under study (Medina *et al.* 2006).

Allelic frequencies of AFLP fragments were estimated using the Bayesian method implemented in AFLP-SURV v.1.0 (Vekemans *et al.* 2002) with the non-uniform prior distributions of allele frequencies option. The Bayesian method of AFLP-SURV produces statistically unbiased estimates of genetic diversity and genetic distances (Zhivotovsky 1999). Allele frequencies were used to estimate overall F_{ST} between host populations and pairwise F_{ST} between host-plants using 100,000 permutations in ARLEQUIN (Excoffier & Lischer 2010). Significance of F_{ST} was estimated with 10,000 permutations. Additionally, Nei's genetic distances between pairs of populations were estimated in AFLP-SURV using 10,000 permutations. Genetic diversity for each population was measured by estimating the number of polymorphic loci and Nei's gene diversity.

Genetic distances between pairs of populations were used for Principal Coordinate Analysis (PCoA) in GenAlEx 6.5 (Peakall & Smouse 2012). An analysis of molecular variance (AMOVA) implemented in GenAlEx was also used to estimate hierarchical genetic

structure within and among populations using host plants and geographic location as source populations. Here, sampling locations were grouped by region, i.e., east versus west Texas (Figure 1), to reflect the potential effect of precipitation on genetic differentiation as outlined by Barman *et al.* (2012). We performed a five-part AMOVA: differentiation (1) among host plants, (2) within host plants, (3) among sampling regions, (4) among sampling locations within regions, and (5) within sampling locations. AMOVA calculates Φ_{PT}, an analogue of F_{ST}, by using a squared Euclidean distance matrix between AFLP fragments. Φ_{PT} is a band-based approach recommended for AFLP data because it does not depend on assumptions that underestimate genetic variability (Lynch & Milligan 1994; Yan *et al.* 1999). Genetic structure was further assessed with the Bayesian model-based clustering algorithm implemented in STRUCTURE v.2.3.4 (Falush *et al.* 2007; Pritchard *et al.* 2000). Clustering in this model was based on the assumption of admixed populations with independent allele frequencies. Sampling source (i.e., host plant) was used as prior information to assist the clustering method. A burn-in period of 10,000 and a run length of 10,000 Markov Chain Monte Carlo (MCMC) iterations were performed for 20 runs for clusters (K) ranging from 1 to 14. Delta K (ΔK) was estimated based on Evanno *et al.* (2005) to select K with the highest probability of predicting population structure in the dataset. Given that populations with stronger structuring may hide structuring in other populations (Evanno *et al.* 2005), STRUCTURE was first run on the whole dataset, then re-run after consecutively removing populations with distinct clusters (i.e. scurvy mallow and horsemint).

Results

The primer pair used in this study yielded a total of 62 AFLP loci. A SESim statistic of 0.011 indicated that the number of loci and individuals in our dataset were sufficient to describe the population clustering pattern produced by CFH in our study. The percentage of polymorphic loci per host plant ranged from 45% to 79% with scurvy mallow (SM) and both cotton (CT) and primrose (EP) yielding the lowest and highest polymorphisms, respectively (Table 2). Estimates of Nei's genetic diversity were similar across host-plants with an average of 0.06 (S.E = 0.003). Overall, genetic differentiation of host-plants based on F_{ST} was low, but significant (0.03; $p = 0.01$). Pairwise F_{ST} values among host plants indicate that genetic differentiation was either absent or low among most hosts (Table 3). CFHs on scurvy mallow (SM), however, were genetically distinct when compared with all other hosts, with pairwise F_{ST} estimates ranging from 0.29 to 0.32. Likewise, differentiation of horsemint (HM) was significantly different from other hosts, with pairwise F_{ST} ranging from 0.06 to 0.30 (Table 3).

When host-plants from all locations were grouped together in the AMOVA analyses genetic differentiation among host-plants explained low but significant variation in CFH (7%), whereas much of the variation was explained within host-plants (i.e., 93%). When sampling locations were grouped by region (i.e., east versus west Texas (Figure 1)) to reflect the potential effects of precipitation on genetic differentiation (Barman et al. 2012), AMOVA detected 96% variation within locations while variation among regions and variation among locations within regions explained only 0% and 3%, respectively (Table 4).

Table 2. Genetic diversity indices of CFH from different host-plants. Host-plants are abbreviated by their common names (see Table 1 for taxonomic information).

Host plant	N*	PLP†	Hj‡ (SE)
OP	19	72.6	0.15 (0.01)
WR	19	75.8	0.17 (0.01)
EP	20	79	0.18 (0.01)
VB	18	73	0.17 (0.01)
CT	14	79	0.14 (0.01)
SM	18	45	0.18 (0.02)
OC	20	77	0.17 (0.01)
TC	19	68	0.12 (0.01)
SC	8	66	0.17 (0.02)
CH	16	66	0.18 (0.01)
HM	19	53	0.13 (0.01)
SN	18	65	0.13 (0.01)
PP	23	74	0.15 (0.01)

* Number of samples
† Proportion of polymorphic loci at the 5% level
‡ Expected heterozygosity under Hardy Weinberg genotypic proportions (or Nei's gene diversity).
Total FST = 0.03

Table 3. Pairwise F_{ST} estimates of host-associated CFH populations. Host plants are abbreviated by their common names (see Table 1 for taxonomic information).

Host plant	CT	TC	VB	CH	HM	SN	OC	OP	EP	PP	WR	SM	SC
CT	-												
TC	0.02	-											
VB	0.04	0.05	-										
CH	0.05	0.06	0.05	-									
HM	**0.08***	**0.09**	**0.09**	**0.11**	-								
SN	0.03	0.01	**0.06**	**0.06**	**0.09**	-							
OC	0.02	0.04	0.03	0.03	**0.10**	0.05	-						
OP	0.02	0.02	0.04	0.02	**0.06**	0.02	0.02	-					
EP	0.03	0.02	0.02	0.02	**0.08**	0.03	0.02	0.01	-				
PP	0.04	0.03	**0.05**	0.04	**0.09**	0.03	0.02	0.02	0.02	-			
WR	0.04	0.04	0.04	0.02	**0.10**	**0.06**	0.03	0.02	0.01	0.04	-		
SM	**0.32**	**0.30**	**0.29**	**0.32**	**0.30**	**0.30**	**0.30**	**0.30**	**0.29**	**0.31**	**0.30**	-	
SC	0.08	**0.10**	0.06	0.05	**0.16**	**0.11**	0.05	0.06	0.05	**0.08**	0.08	**0.35**	-

*Values in bold represent significantly different F_{ST} estimates at 0.05 significance level.

Genetic structure was further investigated with the Bayesian-based clustering algorithm in STRUCTURE. Using the complete dataset with individuals from all host-plants across sampling locations, ΔK (Evanno *et al.* 2005) detected 4 genetically distinct genetic origins (Figure 2). In agreement with our other analyses, the scurvy mallow cluster was genetically distinct from individuals collected from all other hosts, with a high probability (approximately >99%) of individual assignment. Individuals from the other host-plants displayed a mixed genotype that varied in relative composition on different host-plant species (Figure 2). A separate analysis in STRUCTURE using only nymphs (from 8 host-plants on which nymphs were sampled) also differentiated CFH on scurvy mallow from those collected from other hosts. However, ΔK for nymphs indicated only 3 genetic origins (Figure 3). Interestingly, nymphs from purple prairie (PP) belonged to only 1 genetically distinct population while adults on this plant belonged to 3 populations. Finally, principal component analysis (PCoA) 1 and 2 explained 82.6% of the genetic variation of CFH (Figure 4). PCoA 1 separated CFHs from scurvy mallow (SM) and horsemint (HM) from CFHs collected from every other host whereas PCoA 2 separated only CFH from horsemint.

Table 4**.** AMOVA results for host-associated CFH populations. AMOVA was partitioned based on the amount of variation: (a) among host plants, (b) within host plants, (c) among regions considering east vs. west Texas as distinct regions (d) among counties in west and east Texas, and (e) within locations sample in each county.

Source of variation	D.f.	SS	Estimated variance	Percent (%) variation	Φ statistic	P value
Host plants						
(a) Among host plants	12	232.21	0.72	8	PT=0.08	0.01
(b) Within host plants	188	1541.20	8.20	92		
Sampling sites						
(c) Among regions	1	15.51	0.03	0	RT=0.00	0.14
(d) Among locations within region	9	122.11	0.28	3	PR=0.03	0.00
(e) Within locations	188	1614.30	8.59	96	PT=0.04	0.00

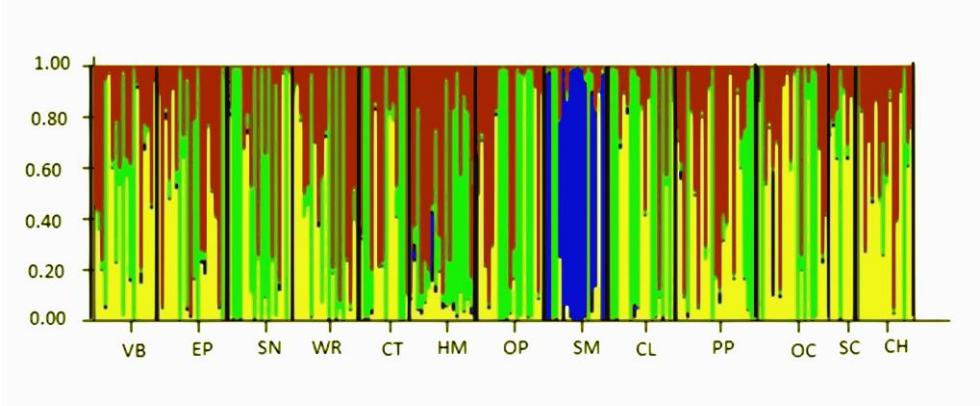

Figure 2. Structure output for both adults and nymphs of CFH. Delta K (ΔK) = 4 from individuals collected from 13 host-plants. Host-plants abbreviated by their common names (see Table 1) are indicated below and separated by black bars. Each colored bar represents an individual CFH with the proportion of color corresponding to the probability that an individual is a member of a particular cluster.

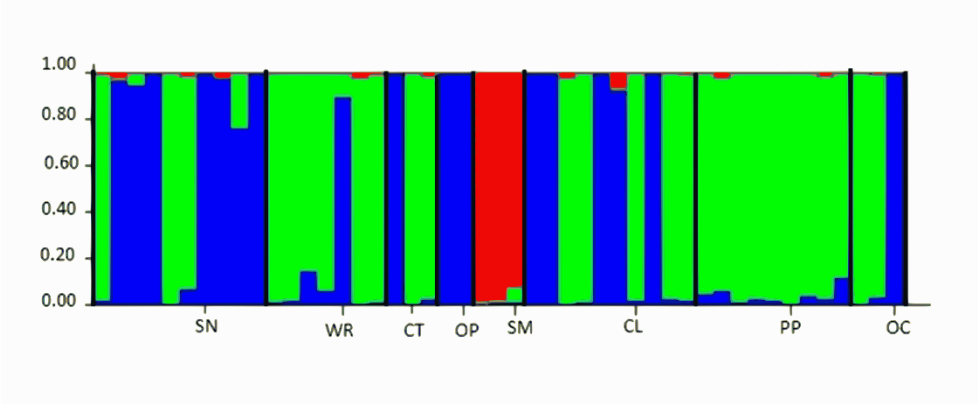

Figure 3. Structure output for CFH nymphs. Delta K (ΔK) = 3 from individuals collected from 8 host plants. Host-plants abbreviated by their common names (see table 1) are indicated below and separated by black bars. Each colored bar represents an individual CFH with the proportion of color corresponding to the probability that an individual is a member of a particular cluster.

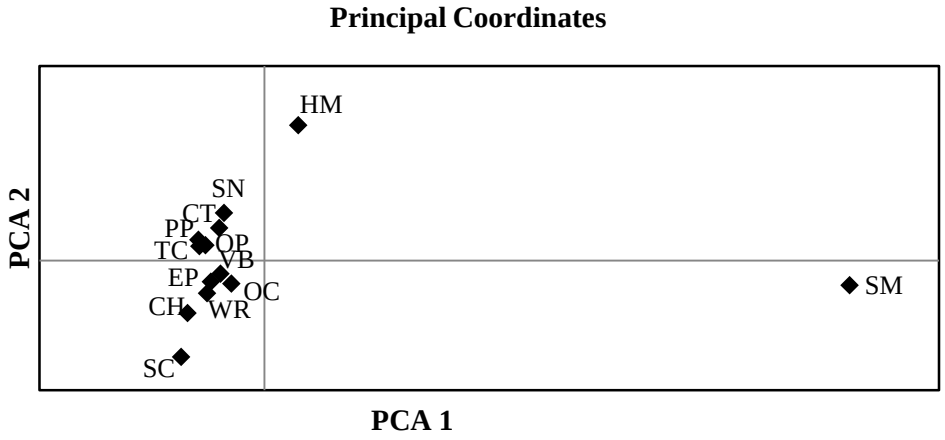

Figure 4. Principal coordinates for CFH associated with 13 host-plants. Host-plants are abbreviated by their common names and denoted by filled diamonds. PCA1 explains 63.8% of the variation; PCA2 explains 18.8%**.**

Discussion

Given that HAD is known to occur in the CFH (Barman et al., 2012) and that it has an extensive host plant range of over 160 plants, we predicted that expanded sampling for HAD would reveal additional instances of HAD. Our results provided limited support for our hypothesis. CFH exhibited slight, but significant, genetic structuring across multiple host-plant species. Although estimates of genetic differentiation were low, host-plants explained a higher proportion of genetic variation in CFH than geographic location (Table 4). The first study of HAD in the CFH by Barman *et al.* (2012) included only 3 host-plant species. In this study, we expanded the assessment of HAD by analyzing CFHs collected from 13 host-plant species. Results from our study identified 4 genetically distinct populations of CFH associated with 13 host-plant species (Figure 2), of which two were distinctly associated with a specific host-plant. CFHs from scurvy mallow were genetically distinct from CFHs from any other host (Figures 2, 3 and 4, and Table 3). CFHs collected from horsemint were also differentiated when compared with CFHs collected from other host-plants (Figure 4 and Table 3). However, CFH genotypes on horsemint although differentiated, were not unique to horsemint (Figure 2).

In their study, Barman *et al.* (2012) found that CFH associated with horsemint showed strong HAD but the pattern of differentiation was typical of a "geographic mosaic of HAD". In other words, horsemint populations in west Texas displayed a strong pattern of HAD, but in east Texas HAD was absent. Barman *et al*. (2012) speculated that the patchy distribution of horsemint in west Texas relative to the plant's almost continuous distribution in east Texas could potentially explain the differential presence of HAD in these two regions. Our study examined CFH in west Texas populations not only on horsemint and cotton (as in

the Barman *et al.* 2012 study), but also on other uncultivated plants (e.g., silverleaf nightshade, purple prairie, old plainsman, and common horehound), revealing that CFH genotypes found on horsemint were not uniquely associated with this plant species. That is, the horsemint genotype characterized by Barman *et al*. (2012) was also present in other uncultivated plant species. Both Barman *et al*. (2012) and this study examined adults on horsemint. However, future studies should genetically characterize nymphs (see below for further discussion about nymphs) on horsemint and compare them with nymphs from uncultivated vegetation. Comparing genetic population structure of nymphs versus adults from different host-plant species will increase our understanding of CFH host-plant fidelity, mating and dispersal behavior.

Populations on scurvy mallow were genetically distinct from those on the other hosts tested in this study. The scurvy mallow plants we sampled were in close proximity with oldplainsman, silverleaf nightshade, common horehound, and purple prairie. In order for host-related selection to cause divergence among populations in such close proximity, there has to be sufficient reduction in gene flow among populations associated with these different host-plants. If divergent selection on CFH associated with scurvy mallow is linked to mating and/or oviposition preference, then selection may have favored assortative mating on scurvy mallow facilitating HAD.

In pea aphids, HAD was first reported in populations associated with alfalfa and red clover (Leonardo & Muiru 2003; Via 1999). Later, another distinct lineage of pea aphids was found on populations associated with pea and faba bean (Carre & Bournoville 2003; Frantz *et al.* 2006; Simon *et al.* 2003). After testing for HAD on 19 widely distributed plants, Peccoud *et al.* (2009) found 11 distinct host-associated lineages of pea aphids in Western Europe. In

the case of the highly polyphagous CFH, when Barman *et al.* (2012) tested HAD on 3 host-plants, they detected 1 host-associated lineage. In our study, extending the number of host-plants did not dramatically increase the incidence of HAD, suggesting that compared to pea aphids, HAD is rather uncommon in CFH. The scarcity of HAD in CFH compared to pea aphids may at least partly be explained by differences in their mode of reproduction.

It has been proposed that parthenogenesis may increase the incidence of HAD (Medina 2012). In fact, several HAD case studies involve parthenogenetic organisms like pea aphids (Via 1999), grain aphids (Simon *et al.* 1999; Vialatte *et al.* 2005), yellow pecan aphids (Dickey & Medina 2010), western flower thrips (Brunner *et al.* 2004; Brunner & Frey 2010), and eriophyid mites (Evans *et al.* 2013). However, HAD also occurs in sexually reproducing insects like grasshoppers (Apple *et al.* 2010; Sword *et al.* 2005), green mirids (Hereward *et al.* 2013), fall armyworms (Pashley 1986), and brown tail moths (Marques *et al.* 2014). Unfortunately, in all these cases HAD was tested across only a handful of host-plant species, making it impossible to know whether HAD extends beyond the sampled plants. We predict that HAD in sexually reproducing insect herbivores will parallel the pattern we have found in the CFH. That is, HAD will be present in a rather small proportion of host-plants. On the contrary, HAD in parthenogenetic herbivores is expected to be present in several of their host-plants, as it has already been reported in the pea and cotton aphids (Vanlerberghe-Masutti *et al*., 1998, Ferrari *et al*., 2006, Peccoud *et al*., 2009).

To test whether dispersing adults were confounding the population structure found in this study, we used only nymphs (due to their relatively low dispersability) in a separate STRUCTURE analysis. Although the analysis of nymphs did not dramatically change the overall pattern of HAD in CFH (Figure 3), it made it less “noisy”. Interestingly, nymphs on

purple prairie harbored only one genotype (Figure 2) while adult populations harbored three (Figure 2). All other plants, except for scurvy mallow, supported two genotypes when only nymphs were considered (Figure 3). The differences observed in the analyses of nymph and adult genetic population structure could be explained by adult dispersal among host-plant species.

HAD of CFH populations may have practical implications for pest control. The fact that genotypes found in cotton can also be found in nearby uncultivated vegetation suggests that several native hosts-plants act as sources of CFH in cotton fields. However, some host plant species like scurvy mallow and horsemint harbor CFH genotypes that are genetically distinct and may not contribute to building up pestiferous populations in cotton. This same phenomenon has been observed in wheat where populations of cereal aphid, *Sitobion avenae* F., associated with wild vegetation do not contribute to the build-up of pestiferous populations in wheat (Vialatte *et al.* 2005). Thus, plants like scurvy mallow and horsemint could be considered as plants suitable to use in conservation biological control programs to enhance local CFH natural enemy populations. Interestingly, CFH populations in horsemint have been found to be genetically distinct only in west Texas. Populations of CFH in east Texas are identical to CFH populations in cotton (Barman *et al.* 2012). Geographic variation in the pattern of HAD stresses the need to study pests' population structure across their entire geographic distribution and host-range. Genetic population structure of pest species may inform locally adapted control strategies in area-wide integrated pest management (IPM) programs.

CHAPTER III

LACK OF CORRELATION BETWEEN HOST-ASSOCIATED DIFFERENTIATION (HAD) AND MICROBIOME OF COTTON FLEAHOPPER, *PSEUDATOMOSCELIS SERIATUS* (HEMIPTERA: MIRIDAE)

Synopsis

Through their associations with different host-plant species, insect herbivores may undergo genetic divergence in a process called host-associated differentiation (HAD). While HAD is typically linked to genetic variation encoded within the nuclear or organellar genomes of insects, there is also evidence that bacterial symbionts can influence the evolution of plant-insect interactions. Studies investigating the role of symbionts on HAD, especially at the microbiome level are rare, and when they have been completed, they usually involve only a handful of model organisms such as pea aphids and their associated host-plants. Cotton fleahopper (CFH) (*Pseudatomoscelis seriatus*) provides an interesting model to test how common the correlation between bacterial presence and HAD is. CFH is a sexually reproducing highly polyphagous insect that has undergone HAD on some of its host-plants. I used 454 pyrosequencing of the bacterial 16S rRNA gene to analyze the microbiome associated with CFH adults from cotton (*Gossypium hirsutum*) and horsemint (*Monarda punctata*), in which HAD had been previously detected between. Taxonomic assignment of bacterial sequences yielded 125 OTUs at the genus level, yet there was no correlation between bacterial communities and HAD in CFH. Interestingly, we detected *Wolbachia* in CFH from only one location. Multilocus sequence typing and phylogenetic

analyses indicated that CFH from this location harbor different strains of *Wolbachia* when associated with cotton and horsemint.

Introduction

For many insect herbivores, different populations of a species may specialize on local host-plants (Ehrlich & Raven, 1964). When associated with different host-plant species, insect populations are likely to experience different selection pressures that may result in the formation of host races or genetically distinct populations (Feder *et al*., 1993; Nosil & Crespi, 2006; Pashley, 1986). This phenomenon is commonly referred to as host-associated differentiation (HAD) (Abrahamson *et al.*, 2001; Bush, 1969). Several studies have sought to investigate the phenomenon in a variety of insect species (Althoff *et al.*, 2007; Funk *et al.*, 2002; Heard, 2012; Hernadez-Vera *et al.*, 2010; Medina *et al.*, 2012; Sword *et al.*, 2005). While HAD has been typically linked to genetic variation encoded within the nuclear or organellar genomes of insects, there is increasing recognition that bacterial symbionts may influence the evolution of plant-insect interactions (Ferrari & Vavre, 2011; Ferrari *et al.*, 2012; Medina *et al.*, 2011). This prompts the need to consider the combination of host and symbiont genomes (i.e., the holobiont) as important components experiencing selection (Rosenberg *et al.*, 2010; Rosenberg *et al*., 2009).

Many insects have established symbiotic relationships with bacteria that, in several instances, play critical and often beneficial roles in their development and survival (Brownlie & Johnson, 2009; Douglas, 1998; Gross *et al.*, 2009; Oliver *et al.*, 2010; Scarborough *et al.*, 2005). Mutualistic associations with bacterial symbionts may provide insects with significant fitness benefits including resistance to pathogenic microorganisms, protection against natural

enemies, and broadening their host-plant range (Oliver *et al.*, 2010; Scarborough *et al.*, 2005; Xie *et al.*, 2011).

Some bacterial symbionts may promote HAD in insects (Ferrari *et al.*, 2007; Ferrari *et al.*, 2012; Medina *et al.*, 2011; Tsuchida *et al.*, 2004). The most notable example of the potential link between bacterial association and HAD involves the pea aphid (*Acyrthosiphon pisum*) and its bacterial symbiont, *Regiella insecticola* (Ferrari *et al.*, 2012; Leonardo & Muiru, 2003; Tsuchida *et al.*, 2004). In the United States, two distinct genetic lineages of pea aphids occur on alfalfa (*Medicago sativa*) and clover (*Trifolium pratense*) (Tsuchida *et al.*, 2004; Via, 1999). Pea aphids' association with clover is strongly correlated with the presence of *R. insecticola*, yet the role of *R. insecticola* in explaining the occurrence of HAD remains controversial (Tsuchida *et al.* 2004, Leonardo & Muiru 2003, Ferrari 2004, Russell *et al.* 2013, Simon *et al.* 2003). The effect of *R. insecticola* on the pea aphid's ability to specialize on clover is complex and strongly depends on aphid genotype and the strain of *R. insecticola* they host (Ferrari *et al.*, 2007, Leonardo & Muiru 2003, Tsuchida *et al.*, 2004). This suggests that intraspecific variation even within symbiotic bacteria may explain the variation observed in the ecological benefits certain symbionts seem to confer. In addition, several insect herbivores are not just infected with a single bacterial symbiont, but with multiple bacteria. Interactions among these symbionts could play important evolutionary roles affecting the insects' specialization and genetic differentiation when on different host plants (Brady *et al.*, 2014; Brady & White, 2013; Medina *et al.*, 2011). Unfortunately, studies investigating the role of symbionts on HAD at the microbiome level are rare, and when they have been completed, they usually involve only a handful of model organisms (Ferrari *et al.*, 2007;

Ferrari *et al.*, 2012; Gauthier *et al.*, 2015). Considering that bacterial symbionts are common in insects, it is likely that their role in explaining HAD could be widespread.

A recent study by Medina *et al.* (2011) indicated that variation in the microbiome of pecan leaf Phylloxera, *Phylloxera notabilis,* associated with pecan, *Carya illinoinensis* (Wangenh) and water hickory, *C. aquatica* (Michx f.) correlated with HAD in this insect. Populations of *P. notabilis* on pecan are associated with *Serratia marcescens* and *Pantoea agglomerans* whereas populations on water hickory lacked these bacteria (Medina *et al.*, 2011). Similarly, genetically distinct cowpea aphids, *Aphis craccivora* (Koch), associated with locust, *Robin* sp., had a high prevalence of *Arsenophonus* while populations on alfalfa were almost exclusively infected with *Hamiltonela* (Brady *et al.*, 2014; Brady & White, 2013). It remains to be explored how common the correlation between bacterial presence and HAD is across a broader number of insect taxa.

The cotton fleahopper (CFH), *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae) provides an interesting model to investigate the role of bacterial communities on HAD. CFH is a generalist insect that is able to feed on at least 160 plant species in 35 families of both managed crops and unmanaged wild plants (Snodgrass *et al.*, 1984). This insect shows HAD when associated with some of its host-plants (Barman *et al.*, 2012, Antwi *et al.*, 2015). HAD of CFH associated with horsemint can be found only at some geographic locations providing a "geographic mosaic of HAD" pattern. Interestingly, horsemint-associated CFH populations at locations with low precipitation displayed HAD while populations at locations with higher precipitation failed to show HAD (Barman *et al.*, 2012). Barman *et al.* (2012) hypothesized that the pattern of HAD they found was the result of differences in host-plant abundance among locations with different precipitation. That is,

HAD was associated with a patchy distribution of horsemint in locations with low precipitation relative to the plant's almost continuous distribution in locations with higher precipitation. In the present study, I test if HAD in cotton fleahopper correlated with differences in CFH microbiome composition. To test this hypothesis I assessed differences in bacterial symbionts between horsemint and cotton associated CFH from locations with high and low precipitation using 454 pyrosequencing.

Materials and Methods

Insect Collections

CFH individuals associated with cotton and horsemint were collected from 5 locations in Texas: College Station, Corpus Christi, Weslaco, San Angelo, and Lubbock (Figure 5). At each location, adults were collected during peak CFH activity on each host-plant when host-plants had reached flowering stages. The insects were collected using standard sweep nets and a motorized blower also known as 'keep-it-simple' sampler (Beerwinkle *et al.* 1997). Ten individuals per host-plant per location were used in this study.

Tag Barcoded FLX 454 Pyrosequencing

DNA was extracted from whole CFHs individually. DNA concentration and quality were quantified using a NanoDrop spectrophotometer (Nyxor Biotech, Paris, France), after which concentrations were standardized to ~100ng/µl. A 1µl aliquot of total DNA per host-plant per sampling location were pooled to characterize bacterial DNA using tag-encoded FLX 454 pyrosequencing (bTEFAP) with the 16S primers, 28F 'GAGTTTGATCNTGGCTCAG' and 519R 'GTNTTACNGCGGCKGCTG', spanning the

variable regions V1-V3. bTEFAP sequencing was carried out by the Research and Testing Laboratory (RTL) (Lubbock, TX).

OTU Clustering and Taxonomic Identification of 16S rRNA Sequences

Pyrosequencing reads were processed with the Ribosomal Database Project (RDP) pipeline (http://pyro.cme.msu.edu/). Raw sequence reads were initially processed by trimming off sequence tags and primers as well as by removing sequences with low quality reads (Cole *et al.* 2009). Remaining sequences were aligned using INFERNAL aligner (Nawrocki *et al.* 2009). A complete linkage clustering method (or the farthest neighbor method), with a 97% sequence identity (ID) threshold was used to cluster sequences to operational taxonomic units (OTUs) (Cole *et al.* 2009). The most abundant and distinctive sequences for each OTU cluster were used as representative sequences for downstream analyses. Chimeric sequences were detected with USEARCH (Edgar 2010) and were excluded from the dataset. Chimeric sequences are typically formed through the fusion of phylogenetically distinct sequences either during PCR or sequencing and often lead to an overestimation of OTUs in 16S rRNA pyrosequencing data (Huse *et al.* 2010; Quince *et al.* 2009; von Wintzingerode *et al.* 1997). Non-chimeric sequences were taxonomically assigned to genus level using the RDP classifier with a 97% bootstrap confidence threshold. A sequence abundance table for the most dominant OTUs was then generated for community analyses.

Community Analysis and Hierarchical Clustering of Sampling Locations

To characterize bacterial diversity and richness of OTUs within and among host-associated CFH populations, we estimated Shannon-Weiner diversity index and Chao 1

richness estimate (respectively). A pairwise (i.e., between host-plants per location) β diversity based on Jaccard index was performed to estimate similarities among bacterial communities. Non-metric multidimensional scaling (NMDS), available in the Vegan package in R (Team 2008), was used to determine whether bacterial communities of CFH clustered with respect to host-plant species.

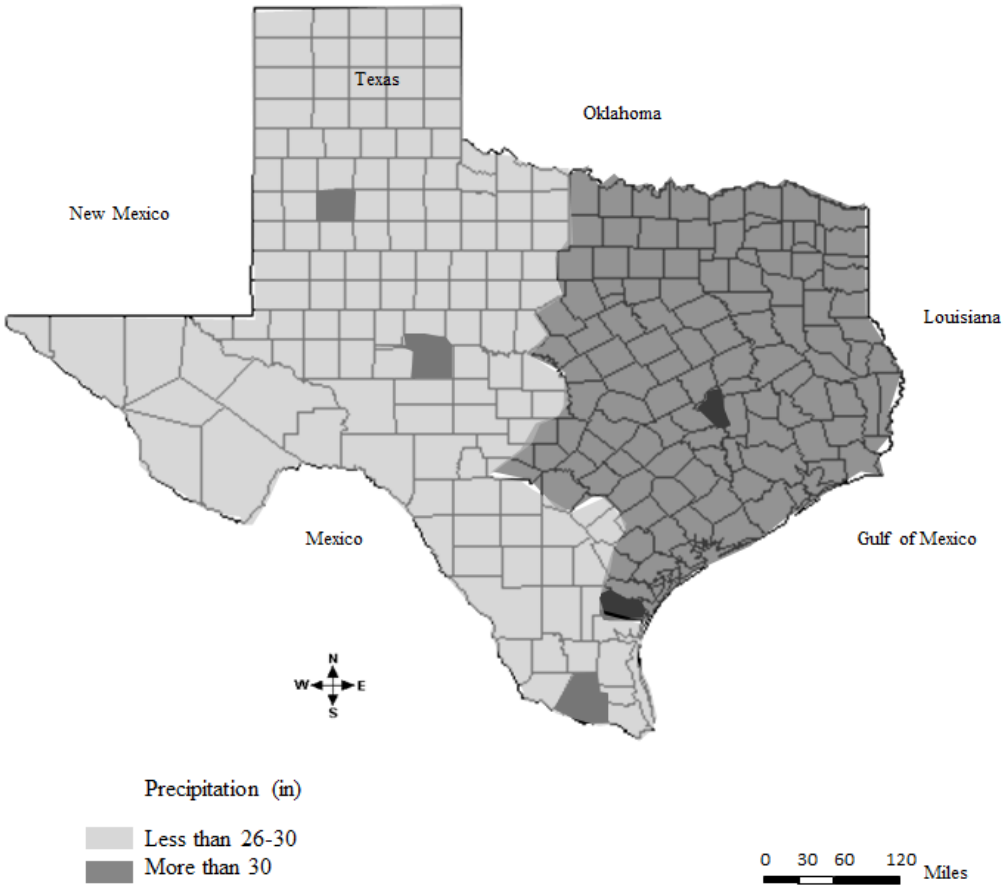

Figure 5. Map indicating sampling locations in Texas. Locations where sampling took place are shaded in dark gray black. The entire state is divided into two regions with respect to annual precipitation as described by Barman *et al.* (2012).

Assessment of HAD on Wolbachia Associated with CFH in Cotton and Horsemint

Interestingly, *Wolbachia* was detected in CFH collected in Weslaco, from both cotton and horsemint, whereas *Wolbachia* was absent in populations from other locations (Table 1). *Wolbachia* has been reported to induce a variety of reproductive phenotypes in insects (Hurst et al., 1999; Werren et al., 2008). Some of these phenotypes have been implicated to cause reproductive isolation among host populations (Engelstadter & Hurst, 2009; Feldhaar, 2011; Shoemaker et al., 1999). Given that CFH populations on horsemint and cotton exhibit strong HAD in Weslaco (Barman et al., 2012), we further assessed if HAD could be reflected in *Wolbachia* associated with CFH in horsemint and cotton. First, to determine the frequency of *Wolbachia* infection in CFH at Weslaco, 10 individuals per host-plant were screened by PCR amplification of the *wsp* (i.e., *Wolbachia* surface protein) gene using primers designed by Jeyaprakash & Hoy (2000). Second, to determine whether infected individuals harbored different strains of *Wolbachia,* 3 multilocus sequence typing (MLST) genes coxA, fbpA, and gatB (Baldo et al., 2006) were characterized using MLST-specific primers and PCR protocols available at the *Wolbachia* MLST website (http://www.pubmlst.org/wolbachia). Consensus sequences of each of the 3 MLST genes were compared with sequences deposited in the *Wolbachia* MLST website to verify *Wolbachia* alleles associated with CFH.

Sequences of each MLST gene associated different host species were selected from the MLST database and aligned with sequences isolated from CFH using ClustalW alignment tool in Mega 6.0 (Tamura *et al.*, 2013). Maximum likelihood trees were generated for each MLST gene using Mega 6.0. Phylogeny for each gene was based on the model T92+G with a bootstrap value of 1,000. Evolutionary models were defined using Mega 6.0 based on the lowest BIC (Bayesian Information Criterion) and AIC (Akaike Information Criterion) values

(Tamura *et al.*, 2013). Trees were viewed and edited in the program FigTree v 1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Pyrosequencing Data

A total of 100 CFH (10 pooled individuals per host-plant per location) were analyzed in this study. Pyrosequencing yielded a total of 53675 sequences. After quality check, 48824 sequences remained with an average sequence length of 357 bp. Taxonomic assignments of clusters identified at 97% sequence similarity resulted in 125 OTUs, at the genus level (Table 5), with the largest OTU comprising 9023 sequences.

Dominant Bacteria Identified within CFH

Bacterial taxa identified to phylum level were dominated by 3 phyla: *Proteobacteria* (65%), *Tenericutes* (26%) and *Firmicutes* (7%) (Figure 6). Operational taxonomic units, identified to the genus level, consisting of 0.3% or more sequence abundance for each sampling unit (i.e., per location per host-plant) are shown on Table 5. Irrespective of the host-plant they were obtained from, CFH from all locations were dominated by three genera: *Spiroplasma*, *Diaphorobacter* and *Pseudomonas*, (Table 5).

Assessment of HAD on Wolbachia Associated with CFH in Cotton and Horsemint

Out of the 20 screened individuals only one individual per host-plant was infected with *Wolbachia*. CFH associated with cotton and horsemint harbored distinct alleles of the FbpA (#406 and #407, respectively) and CoxA genes (#219 and #14, respectively) (Table 6). The FbpA and CoxA alleles found in our study did not correspond to any of the reported alleles on the *Wolbachia* MLST database. In contrast, the GatB allele detected in our study

was identical to allele #9 in the *Wolbachia* MLST database, irrespective of host-plant (Table 6). Maximum likelihood analysis (using 500 bootstrap replicates) of each MLST gene indicates that *Wolbachia* strains in CFH belong to the *Wolbachia* supergroup B. With the exception of the GatB gene, phylogeny of both CoxA and FbpA genes indicate that horsemint and cotton associated CFH *Wolbachia* are more closely related to *Wolbachia* in other insect host species than they are to each other (Figures 7, 8 and 9).

Table 5. Percent abundance of bacterial taxa listed from most to least abundant.

	Location by host-plant									
OTU	[a]CC	CRC	LC	SAC	WC	CH	CRH	LH	SAH	WH
Spiroplasma	43.1	23.7	6.1	62.5	79.6	73.2	33.2	1.6	71.8	79.6
Pantoea	47.0	2.8	3.3	0.0	0.0	6.8	58.1	2.3	12.1	1.1
Lactococcus	0.0	0.0	76.1	14.6	0.0	0.2	0.0	71.9	0.0	0.0
Xanthomonas	0.0	55.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diaphorobacter	2.5	6.0	0.7	2.5	4.8	5.6	3.1	6.7	7.0	4.0
Pseudomonas	1.2	1.4	4.1	0.4	0.5	1.0	0.3	1.1	4.9	0.7
Acinetobacter	0.3	0.7	4.3	0.4	0.3	1.8	0.4	0.0	0.2	0.7
Cloacibacterium	0.5	2.2	0.1	0.1	1.0	0.7	1.0	0.1	0.5	1.0
Serratia	0.0	0.0	1.0	4.1	0.0	0.0	0.0	7.0	0.0	0.0
Arcobacter	0.6	2.0	0.0	0.0	1.4	0.6	0.8	0.4	0.1	0.0
Enterococcus	0.0	0.0	0.0	7.0	0.0	0.2	0.0	0.1	0.0	0.0
Nocardioides	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	4.8
Bacillus	0.2	0.0	0.0	0.0	0.1	2.1	0.0	0.0	0.0	1.4
Propionibacterium	0.1	1.0	0.2	0.2	0.0	0.7	0.4	0.4	0.5	0.7
Arsenophonus	0.0	0.0	0.0	6.3	0.0	0.0	0.0	0.0	0.0	0.0
Exiguobacterium	2.2	0.0	0.0	0.2	0.0	0.3	0.0	0.0	0.0	0.0
Streptophyta	0.2	1.5	0.1	0.4	0.0	0.3	0.4	0.3	0.1	0.4
Rickettsia	0.0	0.0	0.0	0.0	5.7	0.0	0.0	0.0	0.0	0.0
Corynebacterium	0.2	0.3	0.1	0.1	0.1	1.2	0.3	0.1	0.3	0.0
Wolbachia	0.0	0.0	0.0	0.0	4.8	0.0	0.0	0.0	0.0	0.3
Comamonas	0.1	0.3	0.3	0.0	0.1	0.2	0.4	0.4	0.3	0.8
Enterobacter	0.4	0.1	0.8	0.0	0.0	0.5	0.0	0.0	0.0	0.1
Streptococcus	0.0	0.5	0.0	0.2	0.4	0.3	0.5	0.3	0.3	0.2
Staphylococcus	0.0	0.4	0.0	0.2	0.0	0.3	0.2	0.3	0.3	0.2
Stenotrophomonas	0.2	0.1	0.5	0.0	0.2	0.3	0.0	0.3	0.0	0.0
Erwinia	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Escherichia	0.0	0.0	0.1	0.0	0.0	0.8	0.1	0.0	0.0	0.0
Dechloromonas	0.1	0.1	0.0	0.0	0.1	0.3	0.2	0.0	0.1	0.0
Massilia	0.1	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.3
Brenneria	0.0	0.0	0.1	0.0	0.0	0.0	0.0	1.9	0.0	0.0
Weissella	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0
Actinoplanes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7
Methylobacillus	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.4
Curtobacterium	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.4
Phaseolibacter	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Domibacillus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5

Table 5. Continued

	Location by host-plant									
OTU	[a]CC	CRC	LC	SAC	WC	CH	CRH	LH	SAH	WH
Yersinia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0
Aerococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0
Halotalea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0
Halothiobacillus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0

[a]Abbreviations for locations are: San Angelo (SA), Weslaco (W), Lubbock (L), College Station (C) and Corpus Christi (CR). Host-plants abbreviations locations abbreviations (H = horsemint; C = cotton) follow location abbreviations.

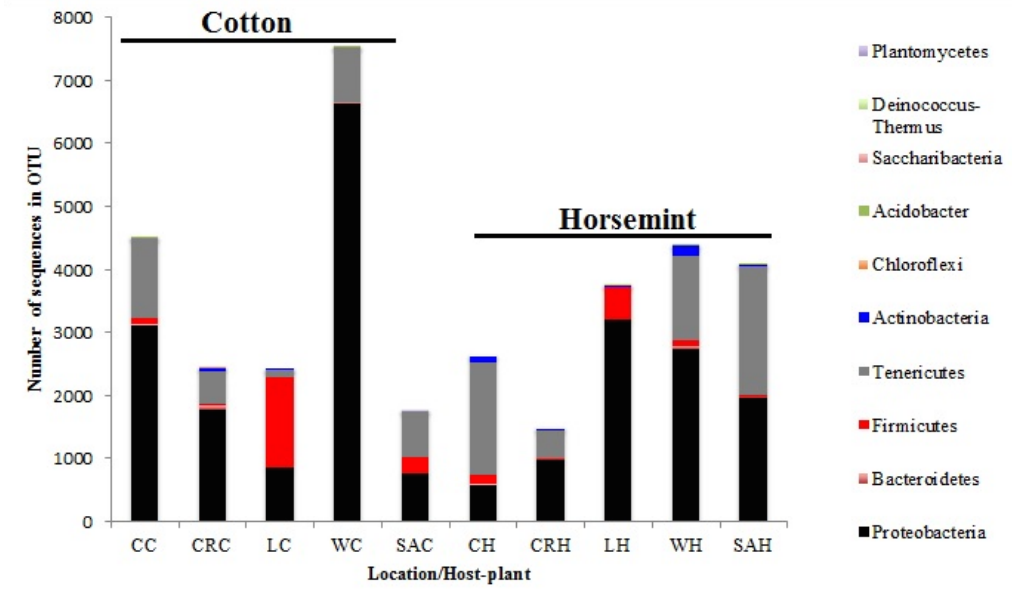

Figure 6. Bacterial taxa detected in CFH from different host-plants. Taxa are represented as the proportion of the total number of sequences from the most dominant bacterial phyla. Abbreviations for locations are: San Angelo (SA), Weslaco (W), Lubbock (L), College Station (C) and Corpus Christi (CR). Host-plants abbreviations (H = horsemint; C = cotton) follow location abbreviations.

Differences Between Bacterial Communities of CFH on Cotton and Horsemint

Bacterial community richness and diversity indexes of CFH ranged from 114.5 – 54.5 and 2.18 – 0.9 (respectively) (Table 7). Overall, there was no difference in the Shannon index of diversity of bacterial communities of CFH associated with horsemint and cotton (mean = 1.80 ± 0.42 and mean = 1.76 ± 0.42, respectively; f = 0.18, d.f. = 1, p = 0.69). Similarly, there was no difference in OTU richness in horsemint (mean = 1.80) and cotton (mean = 1.76) associated CFH bacterial communities (f = 0.0.3, d.f. = 1, p = 0.87).

The structure of bacterial communities based on relative abundances of OTUs across all samples was analyzed by nonmetric multidimensional scaling (NMDS). Overall, bacterial communities of CFH did not cluster by host-plant (Figure 10). Jaccard similarity index (which calculates the proportion of shared OTUs between host-associated CFH populations) and a corresponding hierarchical cluster analysis did not group host-associated CFH populations together (Figure 11). However, there was community clustering by location, where CFH occurring in Corpus Christi and College Station (i.e., east Texas) clustered together, irrespective of host-plant (Figure 10). However, bacterial communities harbored CFH from Weslaco grouped together irrespective of host-plant (Figure 11).

Table 6. Allelic profiles of Wolbachia MLST genes PCR-screened in CFH. Allele numbers indicated by asterisks (*) means the allele is new to the *Wolbachia* database.

Gene	Host plant	Allele #	Supergroup	Length	Host species	Reproductive phenotype
CoxA	Cotton	14	B	402	*Chelymorpha alternans*	Cytoplasmic incompatibility
	Horsemint	219*	B	402	*Pseudatomoscelis seriatus*	-
FbpA	Cotton	406*	B	432	*Pseudatomoscelis seriatus*	-
	Horsemint	407*	B	432	*Pseudatomoscelis seriatus*	-
GatB	Cotton	9	B	369	*Chelymorpha alternans*	Cytoplasmic incompatibility
	Horsemint	9	B	369	*Chelymorpha alternans*	Cytoplasmic incompatibility

Table 7. Richness and diversity estimates of bacterial communities of host-associated CFH.

Location/host-plant	Chao1	Shannon diversity (H')
[a]SAH	114.5	2.18
WH	100	1.71
LH	92.27	1.79
CH	106.11	1.94
CRH	54.1	1.38
SAC	67.07	2.07
WC	90.75	0.9
LC	73.38	1.95
CC	96.23	1.78
CRC	111.8	2.08

[a]Abbreviations for locations are: San Angelo (SA), Weslaco (W), Lubbock (L), College Station (C) and Corpus Christi (CR). Host-plants abbreviations locations abbreviations (H = horsemint; C = cotton) follow location abbreviations.

Figure 7. Phylogeny of the *Wolbachia* MLST gene, CoxA, of CFH. Phylogeny is based on an unrooted maximum likelihood (ML) algorithm in Mega 6.0 (Tamura *et al.*, 2013). Taxa indicated by asterisks (*) are those tested in this study. Bootstrap support values based on 1000 replications are indicated above tree branches.

Figure 8. Phylogeny of the *Wolbachia* MLST gene, FbpA, of CFH. Phylogeny is based on an unrooted maximum likelihood (ML) algorithm in Mega 6.0 (Tamura *et al.*, 2013). Taxa indicated by asterisks (*) are those tested in this study. Bootstrap support values based on 1000 replications are indicated above tree branches.

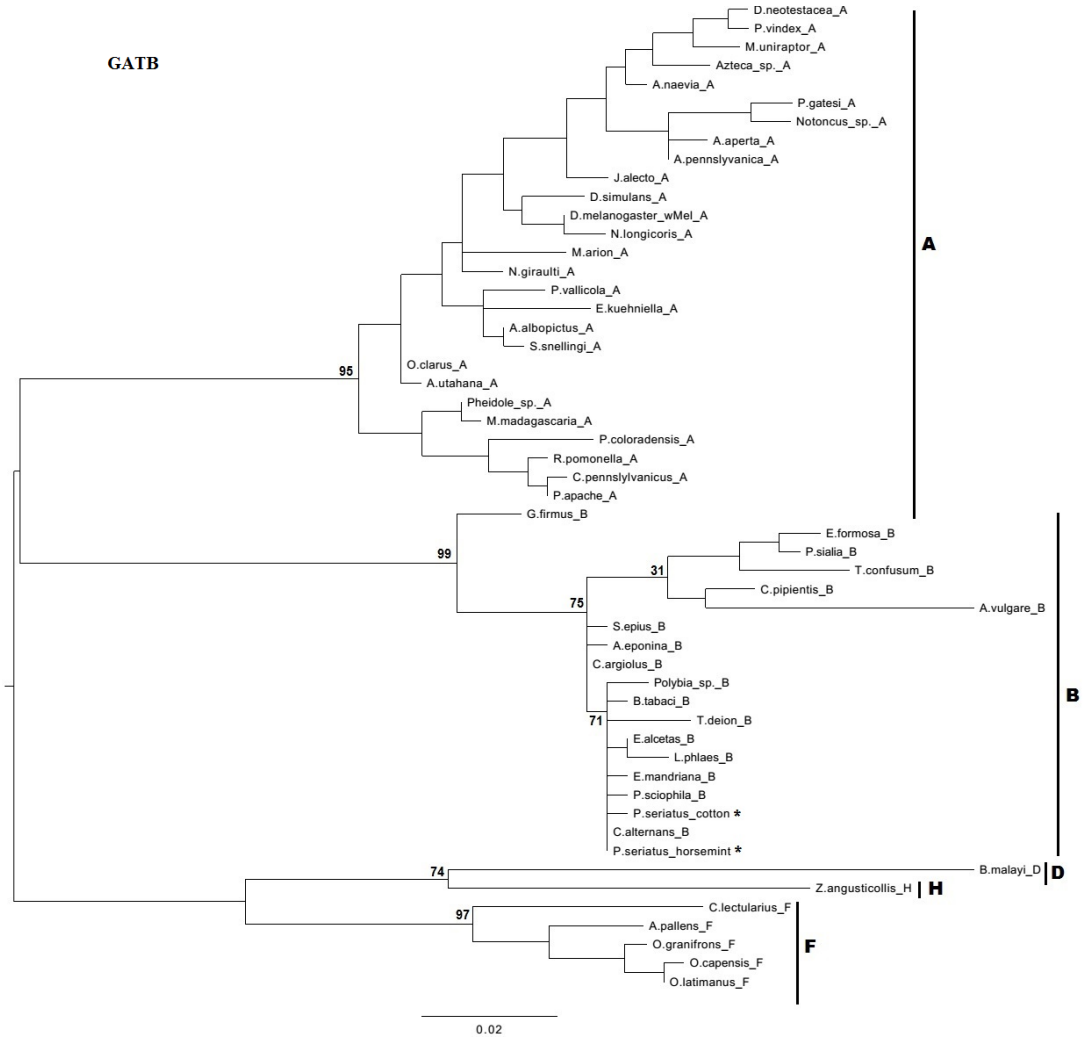

Figure 9. Phylogeny of the *Wolbachia* MLST gene, GatB, of CFH. Phylogeny is based on an unrooted maximum likelihood (ML) algorithm in Mega 6.0 (Tamura *et al.*, 2013). Taxa indicated by asterisks (*) are those tested in this study. Bootstrap support values based on 1000 replications are indicated above tree branches.

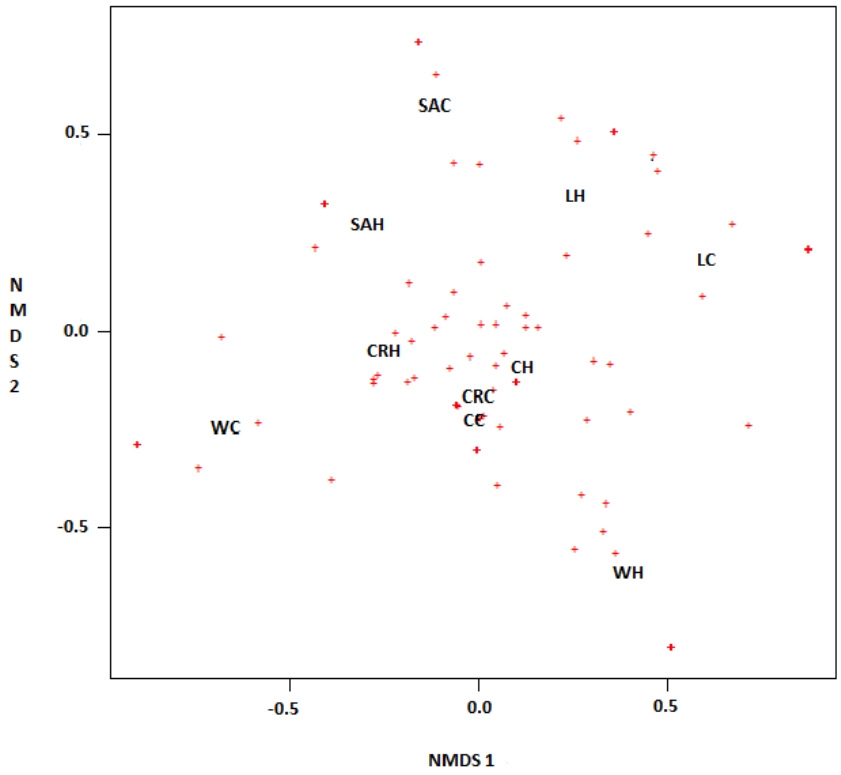

Figure 10. NMDS of bacterial communities of CFH from different host-plants. Dots represent bacterial genera detected in CFH. Abbreviations for locations are: San Angelo (SA), Weslaco (W), Lubbock (L), College Station (C) and Corpus Christi (CR). Host-plants abbreviations locations abbreviations (H = horsemint; C = cotton) follow location abbreviations.

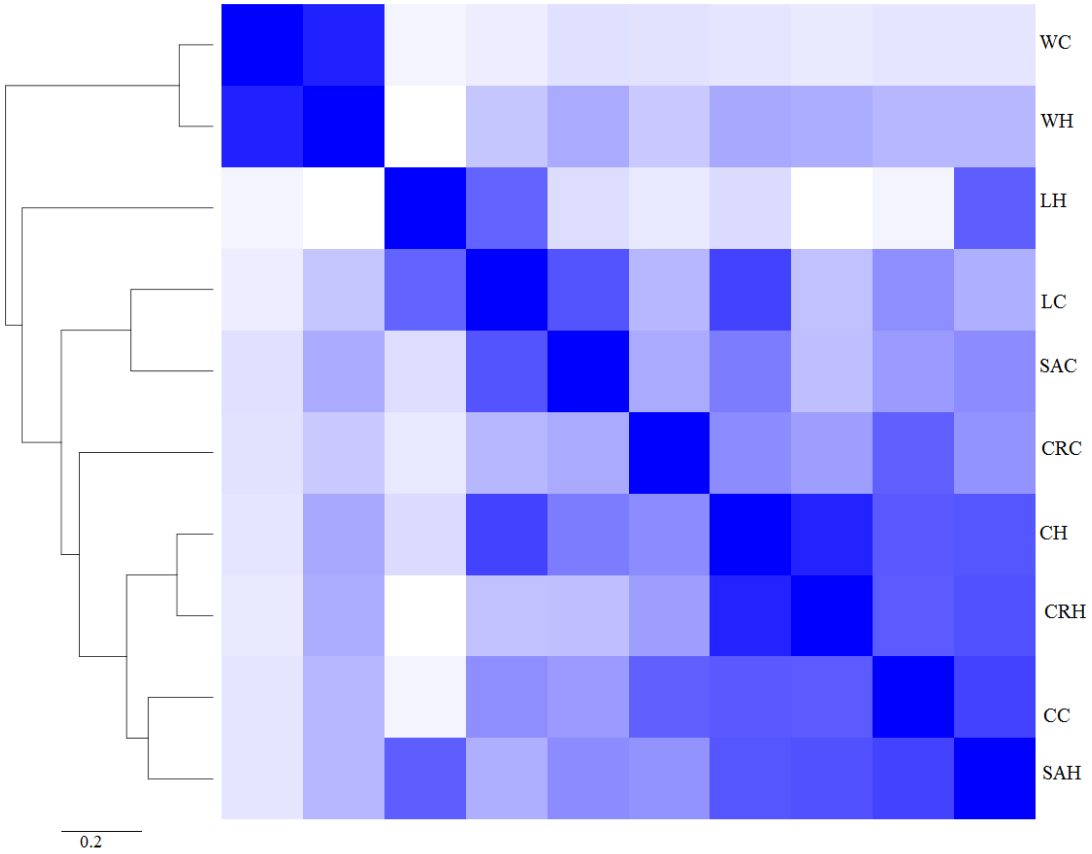

Figure 11. Jaccard similarity of bacterial communities of CFH. Similarities are based on the proportion of shared OTUs between host-associated CFH at 5 locations, and clustered on a heatmap. Abbreviations for locations are: San Angelo (SA), Weslaco (W), Lubbock (L), College Station (C) and Corpus Christi (CR). Host-plants abbreviations locations abbreviations (H = horsemint; C = cotton) follow location abbreviations.

Discussions

Bacterial symbionts play important roles in their host insects, providing ecological benefits such as improved nutrition as well as expand the host range of insect herbivores (Douglas, 1998; Ferrari & Vavre, 201). Recent studies in other Hemipterans have shown that bacterial symbiont composition differs in genetically distinct host-associated insect populations (Brady & White, 2013; Ferrari et al., 2012; Medina et al., 2011). Cotton fleahopper (CFH) associated with two host-plants, horsemint and cotton, show HAD (Barman et al., 2012). Thus, we predicted that variation in the composition of CFH microbiome could correlate with CFH host-associated populations.

Overall, our results did not support our hypothesis. Bacterial communities of CFH from either host-plant did not group together. Additionally, bacterial community diversity did not differ significantly between host-associated populations. However, bacterial communities of CFH occurring in east Texas (i.e., Corpus Christi and College Station), where there is also relatively higher precipitation, clustered together based on NMDS (Figure 10). Even at these locations, a correlation between CFH genotypes and bacterial communities seems unlikely given that CFH at Corpus Christi display distinct genotypes (possibly as a result of local adaptation) from those in College Station, regardless of host-plant associations (Barman *et al*., 2012). Therefore, environmental factors (e.g., local climate) other than direct host-plant influence as well as occasional horizontal transfer of bacteria are likely to be key factors driving bacterial composition among host-associated populations of CFH.

One noteworthy finding from our study is that we detected *Wolbachia* in CFH collected from Weslaco on both host-plants. However, *Wolbachia* was absent in CFH any other locations we sampled. Maximum likelihood analysis of 3 MLST genes indicated that

the *Wolbachia* strains in CFH belong to the *Wolbachia* supergroup B. Other Hemipteran species are also known to harbor *Wolbachia* belonging to the supergroup B (Augustinos *et al.* 2011; Machtelinckx *et al.* 2009; Negri *et al.* 2006). Interestingly, the frequency of infection of *Wolbachia* in CFH was low. After screening all Weslaco individuals used in this study, only 2 out of 20 (i.e., 10%) CFHs were infected. A 10% infection frequency of *Wolbachia* in CFH might be an underestimate given the limited number of individuals examined in this study. However, in another study, a similar number of individuals in two mirid species, *Macrolophus caliginosus* and *M. pygmaeus*, were tested for *Wolbachia* and yielded a 100% infection frequency in both species, including both males and females (Machtelinckx *et al.* 2009). Although we did not account for the sex of individuals used in our study, an overall low infection frequency and the complete absence of *Wolbachia* in CFH from other locations suggest that *Wolbachia* infection in CFH is uncommon or that *Wolbachia* infection in CFH is just beginning.

Interestingly, the MLST genes tested in this study indicated that CFH associated with cotton and horsemint harbored different genotypes of *Wolbachia* (Table 6). For example, based on the phylogeny of the CoxA gene, *Wolbachia* in CFH associated with horsemint seem to be more closely related to *Wolbachia* in *Ostrinia scapulalis*, *Macrosteles fascifrons* and *Apanteles chilonis*, whereas *Wolbachia* in CFH associated with cotton are more closely related to *Wolbachia* of *Polybia sp*., *Diaphorina citri* and *Protocalliphora sialia* (Figure 7). Similarly, a phylogeny based on FbpA gene indicate that *Wolbachia* in CFH associated with horsemint are more closely related to *Wolbachia* in *Lycaeides melissa* and *Leptopilina clavipes*, whereas those on cotton are more closely related to *Wolbachia* in *Glossina palpalis* (Figure 8). Interestingly, the *Wolbachia* strains reported for some of these insect species

induce reproductive phenotypes such as cytoplasmic incompatibility, where embryonic mortality results from mating between uninfected females and infected males or females and males infected with different *Wolbachia* strains (Baudry *et al.* 2003), feminization, where genetic males turns into females (Fujii *et al.* 2001; Sakamoto *et al.* 2008), and parthenogenesis where infected nonfertilized females produce daughters (Pannebakker *et al.* 2005). *Wolbachia* alterations of host reproduction have the potential to accelerate divergence and reproductive isolation of populations with different infection status. Although *Wolbachia* has also been implicated to behave as a mutualistic symbiont of insects, we currently do not know the functional role of *Wolbachia* in CFH, if any. It is important to note that for reproductive isolation induced by *Wolbachia* to persist in host populations, *Wolbachia* prevalence must be high (Charlat *et al.* 2003). Given the low infection frequency of *Wolbachia* in CFH (i.e., Weslaco populations) and the complete absence of *Wolbachia* in CFH populations that also display HAD at other locations (i.e., Lubbock and San Angelo), *Wolbachia* infection in CFH may either be recent or unstable with frequent gains or eliminations. Furthermore, because reproductive manipulations of *Wolbachia* can be costly to host fitness, any host nuclear gene that is capable of eliminating *Wolbachia* infection or repress its effects would persist in the population (Charlat *et al.* 2003). It is possible that CFH may be in conflict with the reproductive effects of *Wolbachia*, as seen in the common pillbug, *Armadillidium vulgare* (Rigaud & Juchault 1993).

Although our study did not detect a correlation between microbiome and HAD, a strong correlation seems to occur in other sucking insects: pecan leaf Phylloxera, cowpea aphids and pea aphids (Brady & White, 2013; Ferrari et al., 2007; Ferrari et al., 2012; Medina et al., 2011; Russell et al., 2013; Tsuchida et al., 2004). The differences observed

between these insects and CFH may, at least, be partly explained by differences in their mode of reproduction. Pecan leaf Phylloxera, cowpea aphids and pea aphids are parthenogenetic insects that can spend multiple generations on their respective host-plants. In addition to their propensity to undergo and maintain HAD on their host-plants (Medina, 2012), parthenogenetic insects are also likely to acquire and maintain microbial symbionts that are either vertically transmitted from mothers to offspring or horizontally transmitted from the insects' niche (Henry et al., 2013). Thus, given the benefits that insect herbivores obtain from bacterial symbionts in their ability to use different, and sometimes novel, host-plants symbionts are likely to be maintained within a host-associated insect population (Douglas, 1998; Oliver et al., 2010). Unlike these parthenogenetic insects, CFH undergoes sexual reproduction with a relatively low frequency of HAD occurring across the insect's host-plant range (Barman et al., 2012; Antwi et al., 2015). Currently, we do not know the mode of bacterial symbiont acquisition in CFH (i.e., whether symbionts are vertically or horizontally transmitted). If the ability to vertically transmit bacterial symbionts increases the likelihood for a host-associated population to maintain a specific microbiome, then mode of reproduction may not be a prerequisite to maintain microbiome. Other sexually reproducing sucking insects like stink bugs are well known for maternal acquisition of bacterial symbionts (Hosokawa et al., 2005; Hosokawa et al., 2007). Yet, we do not know whether these stink bugs have undergone HAD, and whether or not they maintain a microbiome when associated with different host-plant species. Perhaps mere associations with specific host-plants may not be important in structuring bacterial communities within an insect population. Rather the ability to vertically transmit bacterial symbionts while associated with a host-plant is more likely to maintain bacterial communities.

In this study we did not find any correlation between HAD and microbiome of CFH. Although we detected multiple bacteria in CFH, some of which are cotton pathogens that may be potentially vectored by CFH (e.g., *Pantoea ananatis* and *Pseudomonas sp.*) (Bell et al., 2007; Martin et al., 1987), their effects on the life history, fitness and ecology of CFH are currently unknown. A pathogen is transmitted from a vector into a host when the vector is genetically compatible to the survival and complete development of the pathogen while associated with the host (Beerntsen et al., 2000; Geiger et al., 2007). Onion thrips, for example, show HAD on several of its host-plants but only genetically distinct populations on potatoes are able to transmit pathogens to these plants (Brunner et al., 2004; Westmore et al., 2013). The fact that CFH has undergone HAD on some of its host-plants (Barman et al., 2012; Antwi et al., 2015) as well as harbor some of these pathogens may have implications for the insect's competence as a vector of cotton pathogens. If CFH vectors any of the bacterial pathogens detected in this study into cotton, and if CFH's capacity to transmit any of these bacteria into cotton is associated with host-plant source, then it would be important to consider HAD in CFH IPM programs. However, in order to fully understand the ecology and functional roles of most of these bacteria, including vector factors that could be associated with pathogen transmission, more systematic experiments integrating host-associated CFH genotypes and bacteria are needed.

CHAPTER IV

VARIATION IN MICROBIOME DOES NOT EXPLAIN THE PEST STATUS OF THE COTTON FLEAHOPPER

Synopsis

Cotton fleahopper, *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae), is native to the southern United States and widespread throughout regions where cotton (*Gossypium hirsutum*) is commercially grown. It is considered a major cotton pest only in certain cotton-growing regions while in other areas it rarely reaches pest status. Bacterial symbionts associated with sucking insects have been linked to the emergence of pest status of their insect hosts. Here, I test the idea that variation in bacterial communities within *P. seriatus* could correlate with the insect's pest status on cotton. I used 454 pyrosequencing of the bacterial 16S rRNA gene to analyze the bacterial microbiomes associated with 96 *P. seriatus* individuals collected from 8 cotton growing regions in the United States. Results indicate that overall bacterial microbiome composition does not correlate with *P. seriatus* pest status, but bacterial diversity was higher on average at locations where CFH is considered a major pest than at locations where it rarely reaches pest status. Additionally, bacterial communities were structured by location; comprising western and eastern clusters. The observed pattern of microbial clustering generally reflects the regional clustering of *P. seriatus* genotypes previously reported for the insect. This indicates that structuring of both *P. seriatus* genotypes and their associated microbial communities may be affected by similar factors, such as climatic conditions, that vary across these locations.

Introduction

The cotton fleahopper (CFH), *Pseudatomoscelis seriatus,* is an herbivorous insect native to the southern United States and north central Mexico (Knutson *et al.* 2002). It is currently widespread throughout regions where cotton (*Gossypium hirsutum*) is commercially grown in the United States (i.e., the cotton belt) ─ from western California to North Carolina (Greenberg *et al.* 2003; Henry 1991). CFH was first reported as a cotton pest during the 1920s (Reinhard 1926). Early in the growing season, CFH populations rise in cotton fields where both adults and immature insects feed on pinhead-sized floral buds (also called "squares") (Reinhard 1926). Feeding often results in abnormal plant growth, excessive fruit loss, delayed maturity, boll rot disease and fiber loss (Ewing 1929; Martin *et al.* 1987). Damage inflicted by CFH, especially at such critical plant growth stage, makes the CFH an important early season pest of this crop. CFH has only recently been considered a major pest in some cotton-growing regions of the United States (Esquivel & Esquivel 2009). This change in pest status can be explained by improvements in the management of other cotton pests, such as the boll weevil (*Anthonomous grandis grandis)* and cotton bollworm (*Helicoverpa zea)* (Greene *et al.*, 2006). For example, the success of the boll weevil eradication program across most of the United States has resulted in a tremendous decrease in broad-spectrum insecticide inputs in cotton (Duffy & Hishamunda 2001; Moss 1914). Additionally, since the 1990s cotton growers have also utilized *Bt* transgenic cotton, which directly targets Lepidopteran pests such as tobacco budworm (*Heliothis virescens),* cotton bollworm (*Helicoverpa zea*) and others, further reducing insecticide inputs. However, these widespread reductions in pesticide applications have led to the rise of a new suite of sucking

bug pests, including the CFH, that were previously managed indirectly as part of intensive boll weevil and caterpillar control (Greenberg *et al.* 2003; Sansone *et al.* 2009).

Importantly, although CFH is widely distributed throughout the Cotton Belt, it is considered a major pest only in some parts of Texas (e.g., East Texas, Coastal bend, Upper Gulf Coast and the Lower Rio Grande Valley of South Texas), Oklahoma, and Louisiana (Parker *et al.* 2004; Sansone *et al.* 2009; Suh & Westbrook 2010). In West Texas (e.g., Texas High Plains and Rolling Plains), Arizona, North Carolina, South Carolina and Georgia, CFH is considered only an occasional pest where population densities and damage on cotton fields tend to be lower than in major pest regions (Greene 2015; Minsenmayer *et al.* 1988; Parajulee 2013; Sansone *et al.* 2009; Sterling & Dean 1977). Damage estimates vary by region, but in 2009 damage ranged from ~$3 million to ~$7 million in lost income for cotton producers in the southern United States when CFH population densities reach damaging levels (Williams 2010). Currently, uncertainty exists about why the pest status of CFH is not consistent across cotton-growing regions in the United States. It has been speculated that the abundance and diversity of wild host plant species adjacent to cotton fields may contribute to variation in CFH population densities (Beerwinkle & Marshall 1999; Esquivel & Esquivel 2009). For example, the relatively high annual precipitation and the correspondingly high diversity and abundance of wild hosts in East compared to West Texas correlates with the higher CFH population density in East Texas (Esquivel & Esquivel 2009). On the other hand, geographic variation in pest status may be explained by inherent differences between CFH individuals. For example, population genetic studies show that CFH populations associated with one of its most abundant hosts plants, horsemint (*Monarda punctata*), are genetically distinct from cotton populations in West but not in East Texas (Barman *et al.* 2012). In

addition, genetic studies have shown that CFH populations in cotton show distinct geographic genotypes across the Cotton Belt (Barman *et al.* 2013). Thus, even though CFH is distributed across the Cotton Belt, there is genetic variation among CFH populations from different host plant species at different geographic regions. The potential for variation in CFH associations with endosymbiotic bacteria across different host plant species and geographic regions has not been considered to date.

Insects have established symbiotic relationships with bacteria that, in several instances, play critical and often beneficial roles in their development and survival (Brownlie & Johnson 2009; Douglas 1998; Gross *et al.* 2009). In some cases, bacterial symbionts are obligatory for their insect hosts' survival and development by providing essential nutrients (Douglas 1998, 2003; Hosokawa *et al.* 2009; Tada *et al.* 2011). The obligate symbioses of *Buchnera aphidicola* with aphids, *Wigglesworthia* species with tsetse flies, *Portiera aleyrodidarum* with whiteflies, and *Blochmannia* species with carpenter ants, exemplify the complete dependence of these insects on their primary symbionts (Aksoy & Rio 2005; Baumann *et al.* 1995; Douglas 1998; Schroder *et al.* 1996; Thao & Baumann 2004). In addition, several insect species exhibit mutualistic associations with facultative or secondary symbionts. Unlike primary symbionts, facultative symbionts are not required for the host's survival, although they may provide significant fitness benefits. Examples of such benefits include resistance to pathogenic microorganisms, protection against natural enemies, and broadening the range of suitable host plants (Oliver *et al.* 2010; Scarborough *et al.* 2005; Xie *et al.* 2011; Xie *et al.* 2010). Facultative bacterial symbionts can even provide insecticide resistance in some insect hosts. For example, an insecticide-degrading bacterium

(*Burkholderia*) confers insecticide resistance to the bean bug, *Riptortus pedestris*, in rice fields (Kikuchi *et al.* 2012; Kikuchi & Yumoto 2013).

Several studies indicate that facultative symbionts may promote adaptation of insects to their host plants (Ferrari *et al.* 2007; Ferrari *et al.* 2012; Medina *et al.* 2011; Tsuchida *et al.* 2004). Perhaps the best-studied example is the interaction between host-adapted aphids. Aphids adapted to different host plant species harbor distinct facultative symbionts (Brady & White 2013; Leonardo & Muiru 2003; Tsuchida *et al.* 2004), which in some cases seem to influence host plant choice and performance. Facultative symbionts may also confer pest status to insects. For example, *Megacopta punctatissima* (plataspid bean bug) and *M. cribraria* (kudzu bug) harbor the bacterial symbiont, *Candidatus* Ishikawaela capsulata, which determines whether they will become pests in legume crops (Brown *et al.* 2013; Hosokawa *et al.* 2007). In Japan, *M. punctatissima* is a serious pest on soybeans (*Glycine max*), while *M. cribraria* rarely feeds on this crop (Hosokawa *et al.* 2007). Hosokawa *et al.* (2007) found that the pest status of *M. punctatissima* is determined by the presence of *Candidatus* Ishikawaela capsulata ingested from egg capsules upon hatching. By experimentally exchanging capsule-filled symbionts between *M. punctatissima* and *M. cribraria*, their pest statuses were completely reversed on soybeans. Similarly, *M. cribraria* was not a soybean pest in the United States, until 2009 when it was discovered in soybean fields in Georgia. Within a year, *M. cribraria* spread to most parts of the southeastern U.S. (Simon *et al.* 2003; Zhang *et al.* 2012). Interestingly, the rapid spread of *M. cribraria* as a soybean pest in the United States is correlated with harboring "*Candidatus* Ishikawaela capsulata" (Brown *et al.* 2013). Thus, bacterial symbionts, such as "*Candidatus* Ishikawaela capsulata" seem to be responsible for conferring pest status to stink bugs. There are reports

that bacterial communities within whiteflies (Gueguen *et al.* 2010) and western corn rootworm (Chu *et al.* 2013) may play a role in determining host plant range and host adaptation of the insects. We currently do not know the extent to which either specific bacterial taxa or possibly multiple bacteria within insects' microbiome may influence pest status in general.

Multiple bacterial symbionts have been found within insect microbiomes; however, there is notable individual and geographic variation in infection patterns (Apprill *et al.* 2013; Brady & White 2013; Jones *et al.* 2011; Pan *et al.* 2012; Tsuchida *et al.* 2002). For example, pea aphids harbor multiple facultative symbionts yet under variable environmental conditions (e.g. temperature, precipitation, and host plant) certain symbionts may or may not be present in every single individual (Chen *et al.* 2000; Simon *et al.* 2003; Tsuchida *et al.* 2002). Similarly, chestnut weevil populations (*Curculio sikkimensis*) harbor distinct bacterial symbionts in areas with different climatic conditions in the Japanese archipelago (Toju & Fukatsu 2011). Geographic variation in natural climatic conditions may be exacerbated by human activities such as agricultural practices (e.g., timing of planting, timing and frequency of insecticide application). For example, in Texas, the timing of cotton planting differs by ecoregion. Cotton is planted earlier in South and East Texas and later in West Texas. Additionally, the timing and frequency of insecticide application against CFH differs by growing region (Sansone *et al.* 2009; Slosser 1993). Thus, differences in some of these management practices, coupled with variation in local climatic conditions have the potential to influence the composition of the microbiome CFH may harbor at different cotton-growing regions. If the CFH microbiome has any influence on CFH host range or feeding ecology,

variation in microbiome composition may help explain geographic variation in the pest status of this insect.

Here, we examined whether CFH pest status could be linked to variation in the microbial community harbored by this insect. Our specific objectives were: (1) to characterize the microbiome of CFH, (2) to test whether microbiome diversity correlates with pest status of CFH across the United States cotton belt, and (3) to determine the presence of any primary bacterial symbionts associated with CFH. To the best of our knowledge this is the first study testing the idea that communities of bacteria within an insect may influence its pest status.

Materials and Methods

Study Sites and Sample Collection

Cotton fleahoppers were sampled from eight locations in five states spanning the United States Cotton Belt from east to west: North Carolina (NC), South Carolina (SC), Georgia (GA), Texas and Arizona (AZ) (Figure 12). See Barman *et al.* (2013) for detailed information about sampling localities. Samples were taken from one location per state, except for Texas, where samples were taken from 4 locations (i.e., College Station (Cs), Corpus Christi (Cc), Weslaco (Ws) and Lubbock (Lb)). Based on the geographic variation in CFH pest status across the sampled regions, we refer to Cs, Ws, and Cc as "major pest" locations, and AZ, Lb, GA, SC, and NC as "occasional pest" locations. Insects were sampled from cotton plants on cotton fields located at agricultural research centers, extension research stations and in areas where cotton is extensively cultivated. Sampling took place once, on different days, during the early cotton-growing seasons of our locations to coincide with

"square" development and the peak of cotton's susceptibility to CFH as a pest. That is, CFHs were collected in May of 2010 in GA, SC, and NC and 2012 in Ws, Cc, Cs and June of 2012 AZ and Lb. CFHs were sampled using standard sweep nets and a motorized blower ('keep-it-simple' (KIS)) sampler (Beerwinkle & Marshall 1999). Samples were stored either in 85% ethanol or at -80°C prior to DNA extractions. Because of differences in CFH abundance across sites, 12 adults per population were used in the final microbiome analyses to normalize the extent of sampling across locations.

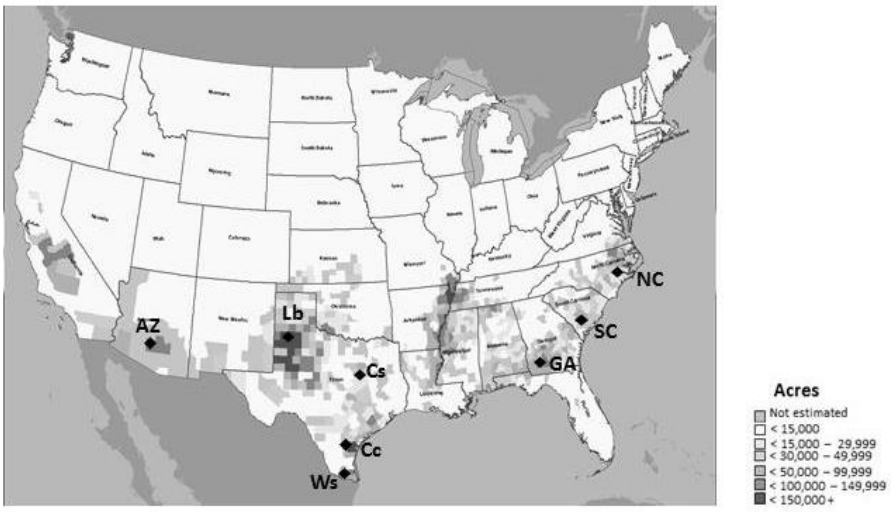

Figure 12. Acreage of cotton planted in the Cotton Belt of the US in 2012. Diamonds indicate cotton-growing regions where cotton fleahoppers (CFH) were sampled: Arizona (AZ), Lubbock, TX (Lb), Weslaco, TX (Ws), Corpus Christi, TX (Cc), College Station, TX (Cs), Georgia (GA), South Carolina (SC), and North Carolina (NC). The figure was adopted from the Planetary Biodiversity Inventory for plant bugs website.

DNA Extraction and Tag Barcoded FLX 454 Pyrosequencing

To avoid contamination by external microbes, insects were washed with 70% ethanol for 1 min, 10% sodium hypochlorite for 5 min, and then rinsed five times with sterilized water (Meyer & Hoy 2008). DNA was extracted from whole insects using a QIAGEN® tissue extraction kit (Valencia, CA) following the manufacturer's protocol. DNA concentration was quantified using a NanoDrop spectrophotometer (Nyxor Biotech, Paris, France), after which concentrations were standardized to ~100ng/ul. 1µl aliquots of total DNA from each individual within each sampling location were pooled. We characterized bacterial DNA using tag-encoded FLX 454 pyrosequencing (bTEFAP) at the Research and Testing Laboratory (RTL) (Lubbock, TX). During library preparation, PCR was performed in triplicate for each sample to minimize random amplicon biases that are commonly detected in sequences obtained from single PCR amplicons (Engelbrektson *et al.* 2010; Haas *et al.* 2011). The 16S primers, 28F 'GAGTTTGATCNTGGCTCAG' and 519R 'GTNTTACNGCGGCKGCTG', spanning the variable regions V1-V3 were used for PCR. Equal volumes of each of the three PCR amplicons were pooled for emulsion PCR and bTEFAP using previously described methods (Dowd *et al.* 2008).

OTU Clustering and Taxonomic Identification of 16S rRNA Sequences

Pyrosequencing reads were processed with perl scripts from Pyrotagger (Kunin & Hugenholtz 2010) to remove reads with 0.2% per-base error probability (bases with < 27 Phred scores). To avoid the problem of unknown identity between non-overlapping sequence segments, remaining sequences were trimmed to a uniform, user-defined length of 243 bp using Pyrotagger. Sequences shorter than 243 bp were discarded and remaining sequences

were aligned using INFERNAL aligner (Nawrocki & Eddy 2013). Sequences were clustered to operational taxonomic units (OTUs) with 97% sequence identity (ID) thresholds. The most abundant and distinctive sequence for each OTU cluster was used as a representative sequence. Chimeric sequences were detected with DECIPHER (Wright *et al.* 2012) and excluded from downstream analysis. Chimeric sequences are typically formed through the fusion of phylogenetically distinct sequences either during PCR or sequencing and often lead to an overestimation of OTUs in 16S rRNA pyrosequencing data (Huse *et al.* 2010; Quince *et al.* 2009; von Wintzingerode *et al.* 1997). Non-chimeric sequences were taxonomically assigned to the genus level using the naïve Bayesian classifier "Multiclassifier" (Wang *et al.* 2007). Because most of our sequences were shorter than 250 bp, and in order to improve classification coverage, we classified OTUs at 50% bootstrap confidence threshold instead of the 80% default threshold in Multiclassifier (Wang *et al.* 2007). All OTUs taxonomically assigned to chloroplast (i.e., comprising 7,545 sequences) were removed from the dataset. We then generated a sequence abundance table for the most dominant taxonomic groups (Table 8).

Community Analysis and Hierarchical Clustering of Sampling Locations

Sequence abundance data (Table 8) was used to estimate the diversity of OTUs at each sampling location. To determine whether our sampling effort was adequate to fully describe bacterial communities within our study samples, we generated rarefaction curves based on the statistical package VEGAN available in R (Team 2008). We used a non-parametric Kruskal-Wallis test in XLSTAT (XLSTAT, New York, NY, USA) to test for

statistically significant differences in bacterial communities in pairwise comparisons between sampling locations.

Sequence abundances of OTUs shared by CFH in at least two locations were used to generate a similarity matrix (based on Euclidean distances between OTUs) and a complete linkage clustering criterion (Huse *et al.* 2010) to cluster OTUs on a heatmap using CLUSTER 3.0 (de Hoon *et al.* 2004). OTUs present at only one site were omitted from the analysis to avoid their potential dilution of the similarity between sites (i.e., to increase the signal-to-noise ratio in the similarity matrix). The output tree was visualized in Tree View (Saldanha 2004). Nonmetric multidimensional scaling (NMDS) was used to determine whether bacterial communities grouped with respect to CFH pest status, i.e., whether communities from "major pest" locations grouped separately from locations where CFH is rarely a pest.

Table 8. Sequence abundance of bacterial genera of CFH from different locations. Numbers in parentheses represent relative abundance of OTUs at each location

OTU	Az	Lb	*Ws	*Cc	*Cs	GA	SC	NC	[a]Rel. abund.
Acetanaerobacterium	0	0	0	0	33 (0.19)	0	0	0	0.07
Achromobacter	0	0	0	0	0	0	181 (17.19)	0	0.40
Acidiphilium	0	0	0	0	0	15 (0.28)	0	0	0.03
Acidovorax	0	0	0	0	50 (0.29)	0	0	1 (0.01)	0.11
Acinetobacter	0	0	0	1 (0.10)	74 (0.43)	0	69 (6.55)	3 (0.03)	0.32
Actinomyces	0	0	0	0	958 (5.61)	0	0	1 (0.01)	2.12
Actinotalea	0	18 (0.22)	0	0	0	0	0	0	0.04
Aerococcus	0	1 (0.01)	0	0	14 (0.08)	0	0	0	0.03
Afipia	0	0	2 (0.05)	0	0	0	0	0	0.00
Albidiferax	0	0	34 (0.86)	0	0	0	0	0	0.08
Alicyclobacillus	0	0	0	0	50 (0.29)	0	0	0	0.11
Allisonella	0	0	7 (0.18)	0	0	0	0	0	0.02
Altererythrobacter	0	3 (0.04)	0	0	0	5 (0.09)	0	0	0.02
Amnibacterium	0	0	0	0	0	0	93 (8.83)	0	0.21
Anaerococcus	0	13 (0.16)	0	0	0	0	2 (0.19)	0	0.03
Anaerofilum	0	0	0	0	0	0	32 (3.04)	0	0.07
Anaplasma	0	0	0	0	0	1 (0.02)	0	0	0.00
Anoxybacillus	0	0	0	0	2 (0.01)	0	0	0	0.00
Arcobacter	0	0	0	0	0	2 (0.04)	0	0	0.00
Arsenophonus	0	0	0	0	0	0	0	3 (0.03)	0.00
Arthrobacter	0	0	0	0	0	2 (0.04)	0	0	0.00
Aspromonas	0	0	0	0	32 (0.19)	0	0	0	0.07
Atopostipes	0	0	2 (0.05)	0	186 (1.09)	0	0	0	0.42
Aurantimonas	0	0	12 (0.30)	0	0	1311 (24.13)	0	8 (0.07)	2.93
Azonexus	0	0	0	0	0	603 (11.10)	0	0	1.33
Bacillus	0	15 (0.18)	0	14 (1.37)	0	104 (1.91)	0	0	0.29
Bdellovibrio	0	3 (0.04)	0	0	0	0	0	0	0.01
Blastococcus	0	0	1 (0.03)	0	0	0	0	0	0.00
Blastochloris	0	0	1 (0.03)	0	0	0	0	17 (0.15)	0.00
Blastomonas	0	1 (0.01)	0	0	0	0	0	0	0.00
Brenneria	0	11 (0.13)	0	246 (24.00)	0	0	0	0	0.57
Brevibacillus	0	0	0	0	0	0	0	39 (0.34)	0.00

Table 8. Continued

OTU	Az	Lb	*Ws	*Cc	*Cs	GA	SC	NC	[a]Rel. abund.
Brevibacillus	0	0	0	0	0	0	0	39 (0.34)	0.00
Brochothrix	0	0	0	0	0	0	2 (0.19)	0	0.00
Burkholderia	0	3 (0.04)	0	0	0	0	0	0	0.01
Butyricicoccus	0	0	0	0	0	8 (0.15)	0	0	0.02
Cellulomonas	0	0	0	0	3 (0.02)	0	0	0	0.01
Chitinophaga	0	0	0	0	0	3 (0.06)	0	0	0.01
Chryseobacterium	0	0	0	0	0	0	6 (0.57)	0	0.01
Citrobacter	0	0	0	0	0	2 (0.04)	0	0	0.00
Cloacibacterium	0	0	5 (0.13)	0	329 (1.93)	20 (0.37)	5 (0.47)	0	0.79
Clostridium	0	0	0	0	89 (0.52)	0	0	4 (0.03)	0.20
Comamonas	0	65 (0.79)	0	0	95 (0.56)	0	0	258 (2.22)	0.35
Conexibacter	0	4 (0.05)	0	0	0	0	0	0	0.01
Corynebacterium	0	25 (0.30)	0	172 (16.78)	0	0	101 (9.59)	2 (0.02)	0.66
Craurococcus	0	0	0	0	0	0	0	20 (0.17)	0.00
Cupriavidus	0	0	0	0	0	0	3 (0.28)	0	0.01
Curvibacter	0	19 (0.23)	0	0	0	0	0	0	0.04
Dechloromonas	0	13 (0.16)	0	75 (7.32)	0	1 (0.02)	0	9 (0.08)	0.20
Deinococcus	0	0	0	0	0	4 (0.07)	27 (2.56)	0	0.07
Delftia	0	0	0	0	0	0	214 (20.32)	0	0.47
Denitrovibrio	0	11 (0.13)	0	0	0	0	0	0	0.02
Desulfomonile	0	0	0	0	0	0	0	6 (0.05)	0.00
Diaphorobacter	0	18 (0.22)	77 (1.94)	0	1249 (7.31)	480 (8.83)	44 (4.18)	0	4.13
Dorea	0	8 (0.10)	0	0	0	0	0	0	0.02
Dysgonomonas	0	0	0	3 (0.29)	0	0	0	0	0.01
Empedobacter	0	0	0	0	0	0	0	9 (0.08)	0.00
Enhydrobacter	0	0	0	0	23 (0.13)	0	0	0	0.05
Enterococcus	0	0	18 (0.45)	0	0	0	0	416 (3.58)	0.04
Erwinia	0	0	2 (0.05)	0	0	9 (0.17)	0	0	0.02
Erysipelothrix	0	0	0	0	10 (0.06)	0	0	0	0.02
Escherichia/Shigella	0	0	5 (0.13)	0	0	0	0	0	0.01
Exiguobacterium	0	0	14 (0.35)	0	0	0	0	0	0.03
Finegoldia	0	0	22 (0.55)	0	0	0	0	0	0.05
Flavisolibacter	0	0	0	0	0	0	59	0	0.13

Table 8. Continued

OTU	Az	Lb	*Ws	*Cc	*Cs	GA	SC	NC	[a]Rel. abund.
Flavobacterium	0	0	0	0	21 (90.12)	3 (0.06)	0	0	0.05
Fontibacillus	0	0	0	0	72 (0.42)	0	0	0	0.16
Friedmanniella	0	0	0	0	0	10 (0.18)	0	0	0.02
Frigoribacterium	0	12 (0.15)	0	0	0	0	0	0	0.03
Fusobacterium	0	0	0	17 (1.66)	0	0	0	0	0.04
Gemmatimonas	0	25 (0.30)	0	0	39 (0.23)	1 (0.02)	0	0	0.14
Gluconobacter	0	7 (0.09)	0	0	0	3 (0.06)	0	1 (0.01)	0.02
Granulicatella	0	0	0	0	0	0	0	1 (0.01)	0.00
Haematobacter	0	0	0	0	0	15 (0.28)	0	0	0.03
Haliscomenobacter	0	6 (0.07)	0	0	0	0	0	0	0.01
Heliothrix	0	0	0	0	190 (1.11)	0	0	0	0.42
Hymenobacter	0	191 (2.32)	0	0	0	0	0	0	0.42
Janthinobacterium	0	0	0	0	0	2 (0.04)	0	0	0.00
Kineococcus	0	0	0	0	46 (0.27)	0	0	18 (0.15)	0.10
Kocuria	0	0	0	0	46 (0.27)	0	0	0	0.10
Lachnospiracea	0	0	0	0	0	22 (0.40)	0	0	0.05
Lactococcus	2833 (33.7)	7123 (86.50)	6	0	0	0	0	0	22.04
Leminorella	0	0	0	0	0	1 (0.02)	0	0	0.00
Leucobacter	0	0	1 (0.03)	0	0	0	0	0	0.00
Leuconostoc	0	0	0	0	1 (0.01)	0	0	0	0.00
Luteibacter	0	0	0	0	1 (0.01)	0	0	0	0.00
Luteimonas	0	39 (0.47)	0	0	0	0	0	0	0.09
Lysobacter	0	7 (0.09)	0	0	0	0	0	0	0.02
Macrococcus	0	6 (0.07)	0	0	0	0	0	0	0.01
Marmoricola	0	0	0	0	0	0	0	13 (0.11)	0.00
Massilia	0	0	3	0	0	0	0	5 (0.04)	0.01
Methylobacillus	0	39 (0.47)	0	0	0	0	0	0	0.09
Methylobacterium	0	6 (0.07)	180 (4.54)	193 (18.83)	4 (0.02)	65 (1.20)	88 (8.36)	0	1.19
Methylotenera	0	0	0	0	2 (0.01)	0	0	0	0.00
Methyloversatilis	0	0	0	0	7 (0.04)	0	0	0	0.02
Microbacterium	0	0	0	0	0	3 (0.06)	0	14 (0.12)	0.01
Micrococcus	0	0	18 (0.45)	0	12 (0.07)	0	4 (0.38)	0	0.08

Table 8. Continued

OTU	Az	Lb	*Ws	*Cc	*Cs	GA	SC	NC	[a]Rel. abund.
Microlunatus	0	0	0	1 (0.10)	0	0	0	0	0.00
Microvirga	0	0	0	0	12 (0.07)	0	0	0	0.03
Moraxella	0	0	0	0	0	0	0	1 (0.01)	0.00
Naxibacter	0	5 (0.06)	0	0	0	0	0	0	0.01
Nitrobacter	0	0	0	0	0	0	0	1 (0.01)	
Nocardioides	0	2 (0.02)	0	0	3 (0.02)	0	0	0	0.01
Novosphingobium	0	2 (0.02)	2 (0.05)	0	0	0	0	82 (0.71)	0.01
Ornithinimicrobium	0	0	0	0	0	0	1 (0.09)	0	0.00
Ottowia	0	0	0	0	1 (0.01)	0	0	0	0.00
Paenibacillus	0	0	0	0	4 (0.02)	0	0	22 (0.19)	0.01
Paludibacter	0	0	0	0	0	0	1 (0.09)	0	0.00
Pantoea	0	0	7 (0.18)	304 (29.88)	0	0	87 (8.26)	402 (3.46)	0.88
Parabacteroides	0	0	0	0	3 (0.02)	0	0	0	0.01
Paracoccus	0	0	0	0	2453 (14.36)	2 (0.04)	0	0	5.43
Pedobacter	0	0	0	0	0	14 (0.26)	0	0	0.03
Peptostreptococcus	0	0	0	0	23 (0.13)	0	0	0	0.05
Phenylobacterium	2 (0.02)	3 (0.04)	2199 (55.43)	0	0	0	0	0	4.88
Propionibacterium	0	6 (0.07)	43 (1.08)	0	0	2 (0.04)	0	0	0.11
Proteiniphilum	0	0	0	0	44 (0.26)	1 (0.02)	0	0	0.10
Pseudomonas	0	9 (0.11)	1 (0.03)	0	1006 (5.89)	9 (0.17)	20 (1.90)	95 (0.82)	2.31
Pseudonocardia	0	36 (0.44)	0	0	0	0	0	0	0.08
Ralstonia	5556 (66.1)	31 (0.38)	0	0	73 (0.43)	62 (1.14)	0	0	12.66
Rhizobium	0	18 (0.22)	0	0	0	0	0	0	0.04
Rhodococcus	0	0	0	0	0	0	0	7 (0.06)	0.00
Rickettsia	0	0	0	0	3 (0.02)	0	0	0	0.01
Rubellimicrobium	0	11 (0.13)	0	0	0	0	0	0	0.02
Rubrobacter	0	0	0	0	13 (0.08)	0	0	0	0.03
Rudanella	0	0	0	0	7 (0.04)	0	0	3 (0.03)	0.02
Salinibacter	2 (0.02)	0	0	0	0	0	0	0	0.00
Sanguibacter	0	2 (0.02)	0	0	0	0	0	0	0.00
Sedimentibacter	0	0	0	0	6	0	0	0	0.01
Sanguibacter	0	2 (0.02)	0	0	0	0	0	0	0.00
Sedimentibacter	0	0	0	0	6	0	0	0	0.01

Table 8. Continued

OTU	Az	Lb	*Ws	*Cc	*Cs	GA	SC	NC	[a]Rel. abund.
Sediminibacterium	0	4 (0.05)	0	0	0	0	0	0	0.01
Serpens	0	17 (0.21)	0	0	8607 (50.38)	0	0	0	19.08
Serratia	0	0	976 (24.60)	0	0	0	0	10134 (87.20)	2.16
Shewanella	0	0	0	0	0	80 (1.47)	0	0	0.18
Shinella	0	61 (0.74)	0	0	0	0	0	0	0.13
Sodalis	1 (0.01)	0	0	0	0	0	0	0	0.00
Skermanella	0	0	0	0	0	67 (1.23)	0	0	0.15
Sphingobium	0	0	3	0	1 (0.01)	9 (0.17)	0	0	0.03
Sphingomonas	0	1 (0.01)	21 (0.53)	0	0	2452 (45.12)	1 (0.09)	6 (0.05)	5.47
Sphingopyxis	0	0	8 (0.20)	0	43 (0.25)	0	0	0	0.11
Spiroplasma	0	0	0	0	523 (3.06)	11 (0.20)	0	3 (0.03)	1.18
Sporichthya	0	1 (0.01)	0	0	0	0	0	0	0.00
Sporobacterium	0	5 (0.06)	0	0	0	0	0	0	0.01
Staphylococcus	0	9 (0.11)	8 (0.20)	0	202 (1.18)	14 (0.26)	0	0	0.52
Stenotrophomonas	2 (0.02)	0	0	0	0	0	15 (1.42)	0	0.04
Streptococcus	5 (0.06)	18 (0.22)	16 (0.40)	0	383 (2.24)	1 (0.02)	0	14 (0.12)	0.94
Streptomyces	0	31 (0.38)	33 (0.83)	0	33 (0.19)	0	0	0	0.21
Subdoligranulum	0	0	0	0	0	3 (0.06)	0	0	0.01
Sulfurospirillum	0	0	0	0	0	9 (0.17)	0	0	0.02
Tanticharoenia	0	0	0	0	0	0	0	3 (0.03)	0.00
Thauera	0	0	0	1 (0.10)	0	0	0	0	0.00
Thermomonas	0	0	0	0	2 (0.01)	0	0	0	0.00
Thermosporothrix	11 (0.13)	0	0	0	0	0	0	0	0.02
Trichococcus	0	0	0	0	0	5 (0.09)	0	1 (0.01)	0.01
Turicella	0	0	0	0	4 (0.02)	0	0	0	0.01
Undibacterium	0	0	242 (6.10)	0	0	0	0	0	0.54
Variovorax	0	273 (3.32)	0	0	0	0	0	0	0.60
Wolinella	0	1 (0.01)	0	0	0	0	0	0	0.00
Zavarzinella	0	0	0	0	0	0	0	3 (0.03)	0.00

* Indicates that CFH is a consistent pest at this location.
[a] Indicates relative abundance of each OTU across all locations.

Results

Pyrosequencing Data

Our goals were to characterize the microbial community within CFH in order to determine whether CFH hosts any primary bacterial symbionts, and to identify whether microbial communities could predict pest status of CFH. A total of 96 CFHs (12 individuals per location) collected from cotton fields within the Cotton Belt of the U.S. were analyzed (Figure 12). Pyrosequencing analysis yielded 82,553 sequences. After quality check, 56,848 sequences remained; with an average sequence length of 243 bp. Thus, clustering at 97% sequence similarity resulted in 156 OTUs (at the genus level) with the largest OTU comprising 11,110 sequences (Table 8).

Dominant Bacteria Identified Within CFH

Six percent of all OTUs identified to phylum were plant chloroplast sequences erroneously classified as *Cyanobacteria* due to the symbiotic origin of these plastids, and these were removed from the analysis. The remaining bacteria were dominated by two phyla, *Proteobacteria* (61%) and *Firmicutes* (31%). For each location, rarefaction curves, which determine how close our sampling effort came to fully describing the bacterial communities within CFH, tended towards saturation (Figure 13).

The bacterial genera detected in this study are shown in Table 8. Of particular interest are five OTUs belonging to the genera *Diaphorobacter*, *Methylobacterium*, *Pseudonocardia*, *Sphingomonas,* and *Streptococcus.* These OTUs were detected in CFH collected from more than 50% of our sampling locations (between five to six locations). No bacterial taxa were consistently present in all eight sampling locations, suggesting that CFH may not be

associated with any primary bacterial symbionts as has been reported in other Hemipteran insects (Douglas 1998, 2003; Hosokawa *et al.,* 2009; Tada *et al.*, 2011; Thao & Baumann 2004).

Differences Between Bacterial Communities of CFH

Cotton fleahopper is considered a major cotton pest at three of eight sampling locations (i.e., College Station (Cs), Weslaco (Ws), and Corpus Christi (Cs)), whereas it seldom reaches pest status in the other sampling locations (i.e., Arizona (AZ), Lubbock (Lb), Georgia (GA), South Carolina (SC), and North Carolina (NC)). Results from both nonmetric multidimensional scaling (NMDS) (Figure 14) and hierarchical clustering analysis, heatmap (Figure 15), indicate that bacterial communities within CFH do not group based on the insect's pest status. Instead, communities from AZ and Lb separated from the other locations (Figures 14 and 15). However, communities in AZ and Lb, where CFH is rarely a pest, do not appear to share a considerable number of OTUs in common, i.e., only <1% of the total number of OTUs occurring at these locations are shared (Tables 7 and 8). Additionally, bacterial communities in NC and Cs separated out from the rest of the locations with 11% OTUs shared by CFH at these sites (Figures 14 and 15). In contrast, bacterial communities in the remaining locations (i.e., Cc, Ws, SC, and GA) grouped together. Thus, with the exception of Cs, the clustering of bacterial communities of CFH generally reflected the regions where CFH was sampled: western region (i.e., AZ and Lb), eastern-most region (i.e., NC), and central region (i.e., Ws, Cc, SC, and GA). Interestingly, in a previous study by Barman *et al.,* (2013), CFH genotypes displayed a geographic clustering of distinct lineages across the geographic regions in our study. The regional clustering of CFH microbes that we

detected in our study partially reflected the genetic clusters of CFH in the cotton belt of the United States.

Bacterial communities of CFH from SC (an occasional pest location) were the most diverse and species-rich, whereas communities in GA (an occasional pest location) were least diverse (by Shannon-Weiner index) (Table 7). On average, bacterial communities were more diverse in insects from pest populations relative to those from occasional pest populations (pest mean = 1.68 (SE = 0.13), occasional pest mean = 1.02 (SE = 0.34). Among the bacterial taxa recovered in this study, we found two genera that may potentially be associated with the pest status of CFH, although we failed to observe a convincing pattern directly linking their occurrence to pest status. These bacteria, *Pantoea* and *Pseudomonas* (both Gammaproteobacteria), are cotton pathogens that are commonly vectored by CFH during feeding (Bell *et al.*, 2007; Martin *et al.*, 1987; Medrano *et al.*, 2009). Although CFH collected from locations where it is considered a major pest harbored *Pantoea* (i.e., Ws and Cc) and *Pseudomonas* (i.e., Ws and Cs), CFH in occasional pest locations such as SC NC, and GAalso harbored *Pantoea* and *Pseudomonas*, respectively (Table 8).

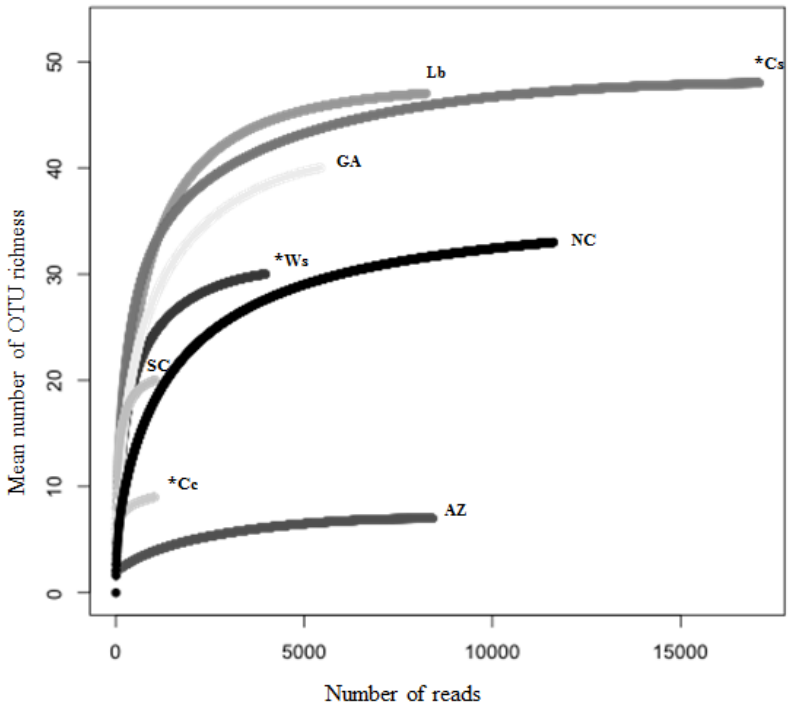

Figure 13. Rarefaction curves of bacterial OTU in CFH. Curves represent mean number of bacterial OTU richness. CFHs were collected from the Cotton Belt: Arizona (AZ), Lubbock, TX (Lb), Weslaco, TX (Ws), Corpus Christi, TX (Cc), College Station, Tx (Cs), Georgia (GA), South Carolina (SC), and North Carolina (NC). Locations with asterisks indicate that CFH is a major pest at that location.

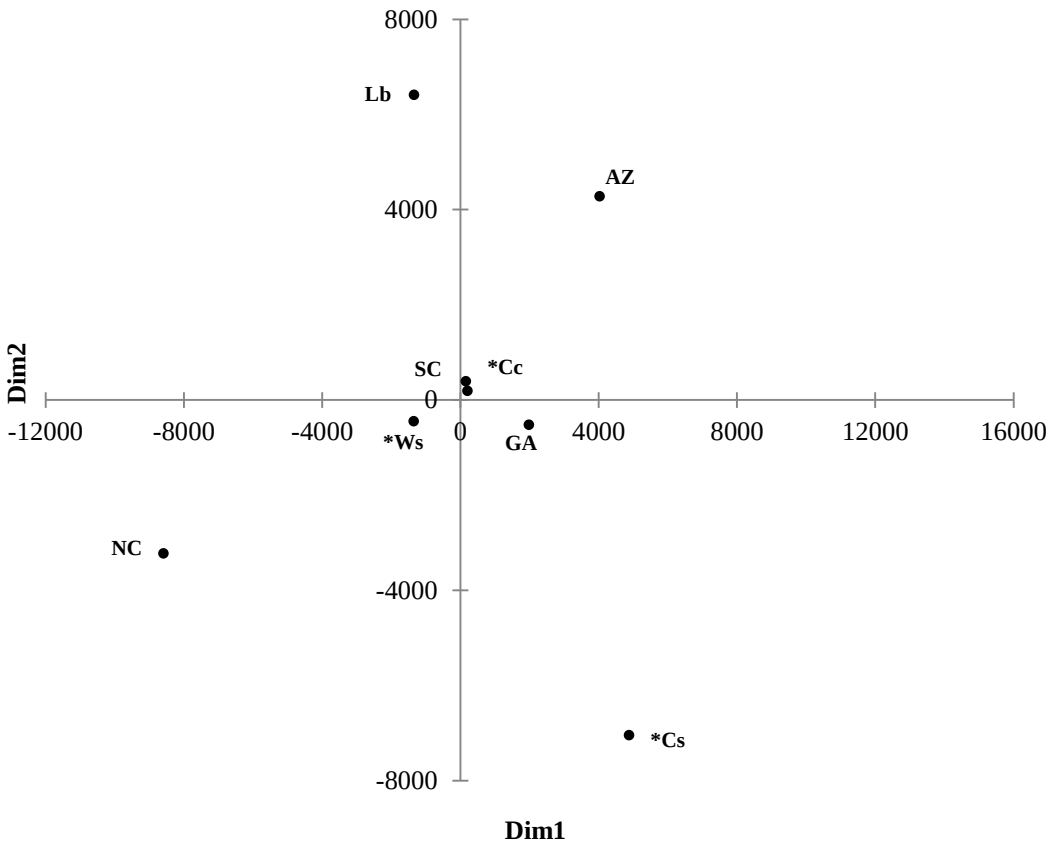

Figure 14. NMDS of bacterial communities of CFH from different locaitons. NMDS is based on a Kruskal-Wallis stress value of 0.14. CFHs were collected from the Cotton Belt: Arizona (AZ), Lubbock, TX (Lb), Weslaco, TX (Ws), Corpus Christi, TX (Cc), College Station, Tx (Cs), Georgia (GA), South Carolina (SC), and North Carolina (NC). Locations with asterisks indicate that CFH is a major pest at that location.

Figure 15. Heat map and hierarchical cluster analyses of CFH bacterial taxa. Graphs are based on the relative abundances of OTUs that occur in two or more locations. CFHs were collected from the Cotton Belt: Arizona (AZ), Lubbock, TX (Lb), Weslaco, TX (Ws), Corpus Christi, TX (Cc), College Station, Tx (Cs), Georgia (GA), South Carolina (SC), and North Carolina (NC). Locations with asterisks indicate that CFH is a major pest at that location. Gray color indicates highly abundant sequence reads and dark to black indicates absence or low abundance of an OTU at a location.

Table 9. Kruskal Wallis p – values between pairwise locations. P - values are based on sequence abundance of bacterial taxa with 500 iterations at alpha = 0.05.

Location	AZ	Lb	*Ws	*Cc	*Cs	GA	SC	NC
AZ	**1**							
Lb	**0.000**	**1**						
*Ws	**0.049**	0.194	**1**					
*Cc	1.000	**0.000**	**0.037**	**1**				
*CS	**0.000**	0.852	**0.018**	**0.000**	**1**			
GA	**0.001**	0.837	0.966	**0.001**	0.299	**1**		
SC	**0.020**	0.538	1.000	**0.013**	0.084	0.998	**1**	
NC	**0.013**	0.468	1.000	**0.009**	0.060	0.998	1.000	**1**

* Indicates that CFH is a consistent pest at this location.
P – values lower than alpha (shown in bold) indicate that bacterial communities are significantly different between pairwise locations.

Table 10. Number of OTUs and diversity estimates of bacterial taxa.

Sampling location	Number of reads	Number of OTUs[a]	Shannon diversity	Species richness
SC	1054	20	2.41	0.65
*Cs	17087	48	1.91	0.39
*Cc	1027	9	1.66	0.31
*Ws	3969	30	1.46	0.49
Lb	8238	47	0.79	0.54
AZ	8412	7	0.66	0.09
NC	11625	33	0.65	0.33
GA	5436	40	0.59	0.56

* Indicates that CFH is a consistent pest at this location.
a Operational taxonomic units (OTUs) were defined with 97% sequence identity (ID) thresholds.

Discussion

We tested the hypothesis that bacterial communities may influence the pest status of CFH. Our results show that overall bacterial microbiome composition did not correlate with CFH pest status (Figure 14). However, bacterial diversity (by Shannon Weiner) in locations where CFH is considered a major pest was higher compared to locations where CFH is rarely a pest (i.e., Lb, AZ, NC, and GA). The only exception was in SC (an occasional pest location), where CFH harbored the most diverse bacterial communities (Table 7). In other insects, diet is thought to be a controlling factor for bacterial diversity, where insects that consume diverse diets harbor more bacterial diversity (Colman *et al.*, 2012; Engel & Moran 2013; Yun *et al.*, 2014). We did not test for gut microbiota specificity in our study, but increased diversity of bacterial communities in CFH in major pest locations may reflect patterns of host plant use by individuals that differ from those in populations where they are only occasional pests. However, the potential for a relationship between increased bacterial diversity and pest status is tenuous given that diversity was highest in the SC population where low CFH numbers and little corresponding damage to cotton plants suggests that CFH was not a pest at the time of sampling (Greene 2015).

Irrespective of pest status, our study indicates that the overall microbiota of CFH is large, dominated by 156 OTUs (identified to the genus level) compared to the microbiota of other insects including *Drosophila* (Chandler *et al.*, 2011; Wong *et al.*, 2011), aphids (Brady & White 2013), whiteflies (Gueguen *et al.*, 2010; Jing *et al.*, 2014) and Psyllids (Jing *et al.*, 2014). The factors explaining the variation observed in bacterial diversity in CFH at different locations remains to be resolved. Identification and comparison of core gut microbes of CFH

from locations that differ in host plant species community composition may help to provide information about the correlation between diet and microbiome variation.

Our sequence library was dominated by two main phyla with sequence similarities (i.e., 97% similarity) to *Proteobacteria* and *Firmicutes*. Our results are consistent with previous findings that *Proteobacteria* and *Firmicutes* are the predominant phyla in insects (Colman *et al.,* 2012; Yun *et al.,* 2014). Additionally, an earlier study characterizing the microorganisms associated with the CFH using culture-dependent microbiological methods confirms our finding that CFH can harbor *Pseudomonas*, potentially pathogenic bacteria vectored by CFH into cotton (Martin *et al.,* 1987) (see below for further discussion). Martin *et al.,* (1987) were the first to study the microbiota of CFH using a relatively shallow microbiological sampling strategy that would not have detected unculturable or low abundance microbes. In contrast, rarefaction curves of bacterial taxa detected using next generation sequencing DNA meta-barcoding approach tended towards saturation for most sampling locations, suggesting that our study came close to fully capturing the bacterial communities present in the CFH samples we analyzed (Figure 8). However, we still may have underestimated the full extent of CFH bacterial diversity given the limitations of small sample size (i.e., 12 individuals per location) and sampling locations. Our use of 12 individuals per location does not reflect the variation in population density of CFH at our sampling sites, particularly in pest populations where CFH can be quite abundant. Low CFH numbers were obtained from locations outside Texas (i.e., non-pest locations), therefore our sample sizes for each location were standardized to avoid finding more rare bacteria at site where significantly more CFH were found. Additionally, we were unable to obtain CFH from

all locations across the cotton belt and may have limited our ability to capture any pattern in microbiome and pest status.

Phytophagous insects are ubiquitously associated with bacterial symbionts, some of which improve insects' nutrition and confer resistance to environmental stressors (Brownlie & Johnson 2009; Douglas 1998; Gross *et al.*, 2009). For shield bugs in the family Plataspidae (e.g., bean bug and the kudzu bug), a specific bacterial symbiont (*Candidatus* Ishikawaella caspulata) makes them pestiferous when associated with leguminous plants (Brown *et al.*, 2013; Hosokawa *et al.*, 2007). Similarly, the western corn rootworm (*Diabrotica virgifera virgifera* LeConte), harbors gut microbiota that seem to improve the insect's resistance to plant defenses from soybean (Chu *et al.*, 2013). Therefore, it seemed reasonable to suspect that some of the observed symbionts would predict the variation in pest status that we see in CFH. Nevertheless, an association of this sort was not apparent for CFH on cotton. Not only was there no clear link between bacterial symbionts and pest status in the CFH, but we also failed to find any OTUs that were consistently present in all samples, suggesting that CFH seems to lack any primary bacterial symbionts.

Despite the lack of correlation between overall microbiota and pest status, we did detect some specific bacterial plant pathogens, *Pseudomonas* and *Pantoea,* that are thought to be vectored by CFH. We do not have any information about the pathogenicity of the specific strains encountered in this study. Infected plants typically show excessive abscission of new growth, including flower buds (or squares) because these pathogens increase levels of ethylene in cotton (Grisham *et al.*, 1987). Ethylene is a phytohormone that regulates fruit abscission under plant stress (Swanson *et al.*, 1975). *Pantoea*-infected cotton plants, following CFH infestation, additionally experience flower bud necrosis that frequently leads

to flower bud and young cotton fruit (boll) abscission (Bell *et al.*, 2007). Although pestiferous and non-pestiferous CFH do not differ dramatically in overall bacteria composition, the presence of key pathogenic bacteria, like *Pseudomonas* or *Pantoea*, could potentially contribute to CFH pest status at some locations. Importantly, the pattern of infection of *Pseudomonas* or *Pantoea* within CFH did not strictly reflect the pest status of CFH in our sampling locations. Interestingly though, CFH at all locations where it is considered a pest (i.e., Ws, Cc, and Cs) were infected with either *Pseudomonas* or *Pantoea*, but neither of these bacteria co-occurred at these locations, except for a *Pseudomonas* singleton detected in Ws (Table 8). Both bacteria were either absent or present together in most locations where CFH was rarely a pest (i.e., AZ, GA, SC and NC). Thus, it seems that absence or co-occurrence of both pathogens in CFH occurring in pest locations may be rare. One possibility is that *Pseudomonas* and *Pantoea*, when present on their own, may increase CFH damage and pest status in cotton but when present together, antagonism or competition for resources (Anttila *et al.*, 2013; Hibbing *et al.*, 2010; Kirisits *et al.*, 2005) may render them inefficient at either colonizing or becoming pathogenic within the plant. In our study, we did not take into account whether or not cotton plants were damaged by CFH during the sampling period. In the future, sampling on cotton should coincide with CFH damage estimates including the presence of pathogens in plant tissues to provide further insights as to whether the presence of these bacteria correlates with CFH damage or pest status.

Other factors such as variation in climatic conditions at our sampling locations may have influenced the geographic variation observed in the CFH microbiome. For example, our western locations (i.e., AZ and Lb) experience lower annual precipitation and higher temperatures (Gleason *et al.*, 2008). Conditions change as one moves eastwards with

increasing annual precipitation and decreasing temperatures (Gleason *et al.*, 2008). The composition of the bacterial microbiome of other insects has been found to vary geographically depending on climatic conditions. For example, Tojuand Fukatsu (2011) have reported differences in infection frequencies of *Wolbachia* and *Rickettsia* in the chestnut weevil (*Curculio sikkimensis*) at localities where weevils experience different atmospheric temperatures. In our study, bacterial communities were structured by location (Figures 14 and 15). We observed a western cluster (comprising of AZ and Lb), a central cluster (comprising of Ws, Cc, SC and GA), and an eastern cluster (comprising of NC). However, bacterial communities in Cs, which geographically lies within the central region, did not group with the central cluster. The pattern of microbial clustering in this study generally reflects the regional clustering of CFH genotypes previously reported by Barman *et al.,* (2013). Overlapping distribution of bacterial communities with host genotypes is not an isolated case in CFH, as other insects such as whiteflies (*Bemisia tabaci*) also show nonrandom distribution of bacterial endosymbionts among whitefly genotypes (Gueguen *et al.,* 2010). It is possible that CFH microbial community composition is directly affected by variation in either climatic conditions or CFH genotypes (or both). However, testing this hypothesis will require manipulative empirical experiments to examine the direct and interactive effects of environmental factors and insect genotype on the composition of the CFH microbiome.

In our study, five OTUs belonging to the genera *Diaphorobacter*, *Methylobacterium*, *Pseudonocardia*, *Sphingomonas*, and *Streptococcus* were commonly detected in CFH. Of the eight locations sampled, CFH collected from five to six locations harbored these bacteria, irrespective of pest status (Table 8). *Sphingomonas* has been previously described as

associated with sucking insects (Jing *et al.*, 2014). *Pseudonocardia* and *Methylobacterium* have been implicated in the regulation of growth of other microbes within insects. *Pseudonocardia* defends against fungal pathogens in fungus gardens of ants (Meirelles *et al.*, 2014), whereas *Methylobacterium* induces the growth of insect-vectored bacterial pathogens (Lacava *et al.*, 2004). However, these interactions could involve complex mechanisms including possible cross talk among microbes and between microbes and host insects that are difficult to dissect based on the identity of individual taxa (this study), some of which are environmental bacteria. Given their prevalence in CFH, additional studies to determine the functional roles or phenotypic effects of these bacteria on CFH are warranted.

To our knowledge, this is the first study to provide a comprehensive description of the microbiome of CFH, a pest that causes significant economic loss to the U.S. cotton industry. Although our study detected multiple microbes associated with CFH, their effects on the life history, fitness and ecology of the CFH are currently unknown. Our study indicates that CFH microbiome composition does not predict CFH pest status. Sample size and limited sampling locations may have limited our ability to detect the influence of bacterial communities on the pest status of CFH. Alternatively, specific bacterial taxa could be better predictors of the variation in CFH pest status as seen in other sucking insects (Brown *et al.*, 2013; Hosokawa *et al.*, 2007; Kikuchi *et al.*, 2012). Although several of the bacteria associated with CFH could be commensalists, others could have some functional roles within CFH and such roles may differ depending on the environmental conditions. The rapid accumulation of microbiome data will likely continue to provide insights into the symbiotic relationships between microbes and insects. It remains to be seen if and how such relationships may be relevant to ecology and pest status of the CFH.

CHAPTER V

FUNGAL ENDOPHYTES, *BEAUVERIA BASSIANA* AND *PURPUREOCILIUM LILACINUM*, POSITIVELY AFFECT THE GROWTH OF SORGHUM AND IMPACT THE BEHAVIOR AND PERFORMANCE OF THE SUGARCANE APHID, *MELANAPHIS SACCHARI*

Synopsis

Plant-fungal endophyte associations can play an important role in plant growth and resistance against insect herbivores. We examined the potential for two fungal endophytes, *Beauveria bassiana* and *Purpureocilium lilacinum* (formerly *Paecilomyces lilacinus*), to improve the development of *Sorghum bicolor* and to control sugarcane aphids (*Melanaphis sacchari*), an invasive pest on sorghum. Seed treatment with *B. bassiana* and *P. lilacinum* increased plant height and fresh biomass 7 days and 30 days after planting. Endophyte treatment also affected the performance of sugarcane aphids, but performance was conditional on both the identity of the endophyte and the part of the plant where the aphids were located. There was no effect, and sometimes a positive effect, of endophyte treatment on aphid performance when aphids avoided lower plant parts and segregated on upper parts. In a subsequent experiment when we controlled for the location of aphids on plants, fecundity was consistently negatively affected on lower plant parts than on upper plant parts, irrespective of treatment type. Interestingly, when aphids did not show preference for any part of the plant, treatment with either *B. bassiana* or *P. lilacinum* significantly reduced aphid performance. Altogether, exploiting the use of beneficial fungal endophytes for improved plant growth and resistance to stress is a promising strategy in agriculture. However, fungal

endophyte-mediated defense against insect herbivores could be a variable phenomenon conditional on the behavioral response of insects on specific plants.

Introduction

Sugarcane aphid, *Melanaphis sacchari*, is a key pest of sorghum widely distributed across many tropical, subtropical, and temperate regions of the world (Singh *et al,*. 2004). In North America, the sugarcane aphid first appeared as a pest on sugarcane fields in Florida in the 1970s, but in the past decade has spread to the southwest of U.S. (White *et al.*, 2001). The sugarcane aphid was first noticed as a pest on sorghum in parts of Louisiana and east Texas in 2013 (Way 2014). It has since spread throughout the south-central U.S. (Armstrong *et al.*, 2015) where it can have negative effects on sorghum development, yield and harvest (Narayana 1975). Currently, the sole method of control is chemical insecticide applications (Singh *et al.*, 2004), although recently developed resistant varieties of sorghum show promise in reducing susceptibility to aphid damage (Armstrong *et al.*, 2015; Sharma *et al.*, 2014). While chemical insecticides may be effective at controlling aphid populations, they are costly and have raised a number of ecological concerns including the evolution of resistant pest strains, and negative effects on environmental and human health (Gore *et al.*, 2013; Kerns & Gaylor 1992; Leach & Mumford 2008; Moores *et al.*, 1998).

In recent years, there has been an increasing interest in the use of artificially inoculated fungal endophytes in integrated pest management (IPM) as a means to reduce insecticide applications on row crops. Fungal endophytes are fungi that colonize a plant without causing visible disease symptoms (Schulz & Boyle 2006). Interactions between fungal endophytes and plants that improve plant growth are indicative of mutualistic

relationships between the two (Elena *et al.,* 2001; Varma *et al.,* 1999). In sorghum, there is evidence that artificial inoculation with fungal endophytes can increase plant height and yield (Reddy *et al.,* 2009). Increased plant vigor has the potential to facilitate a plant's tolerance to biotic stressors such as insect herbivory (Raman *et al.,* 2012).

Clavicipitaceous endophytes (or Class I endophytes) are primarily vertically-transmitted and restricted to grasses where they are well known to confer protection to their hosts by deterring insect feeding and development (Raman *et al.,* 2012; Rodriguez *et al.,* 2009). In contrast, fungi such as *Beauveria bassiana* and *Purpureocilium lilacinum* (formerly *Paecilomyces lilacinus*) are Class II endophytes that can be horizontally-transmitted and occur naturally in a broad range of host plants. In the case of *B. bassiana* and *P. lilacinum*, both fungi are commercially available and typically applied directly to plants or soils as biopesticides for insect or nematode control. However, there is mounting evidence that *B. bassiana* grows endophytically within tissues of a range plant species, including sorghum, resulting in significant reductions in insect performance and populations (Akello *et al.,* 2008; Bing & Lewis 1991; Gurulingappa *et al.,* 2011; Gurulingappa *et al.,* 2010; Lopez *et al.,* 2014; Mantzoukas *et al.,* 2015; Quesada-Moraga *et al.,* 2014; Reddy *et al.,* 2009; Tefera & Vidal 2009). *Purpureocilium lilacinum* has been widely used as a soil inoculant to control soil-borne plant pathogens such as root-knot nematodes (Anastasiadis *et al.,* 2008; Atkins *et al.,* 2005; Kiewnick & Sikora 2006). Only a few studies have tested the potential for *P. lilacinum* to control insect herbivores, and its potential effect as an endophyte has been rarely considered (Imoulan *et al.,* 2011; Wakil *et al.,* 2012). Recent reports indicate that *P. lilacinum* can occur naturally as an endophyte in cotton plants (Ek-Ramos *et al.,* 2013) and its intentional inoculation to cotton has negative effects on the reproduction of cotton aphids,

Aphis gossypii (Lopez *et al.*, 2014). The ability of *P. lilacinum* to establish as an endophyte in other plant taxa and its resulting effects on target herbivores have not been tested.

Endophyte-mediated defense against insect herbivores is a variable phenomenon conditional on the type of endophyte or the insect species. In grasses, fungal endophytes may have different effects on insect abundance and performance depending on the relative position of insects on an infected plant (Christensen *et al.*, 1997; Moy *et al.*, 2000). For example, tall fescue, *Lolium arundinaceum*, associated with the Class I endophyte, *Neotyphodium coenophialum*, compromised the performance of plum aphids, *Rhopalosipum padi*, when the insect was restricted to leaf sheaths relative to leaf blades of plants (Hunt & Newman 2005). A similar phenomenon was reported for bluegrass webworm, *Parapediasia teterrella*, where the insects performed worse on lower plant parts when ryegrass was treated with fungal endophytes (Kanda *et al.*, 1994; Koga *et al*., 1997). Other insects like fall armyworm, *Spodoptera frugiperda*, show variable responses ranging from low survival to better performance on endophyte-treated plants irrespective of location (Raman *et al.*, 2012). In sorghum, treatment with *B. bassiana* has been shown to reduce the performance of sorghum shoot borer (Devi *et al.*, 2001; Reddy *et al.*, 2009). But whether fungal endophytes differentially affect herbivore performance on endophyte-treated sorghum, with respect to an herbivore's position on the plant is not known.

In this study we evaluated treatment effects of *B. bassiana* and *P. lilacinum* on the growth of sorghum and their effects on the sugarcane aphid. Our specific objectives were to: (1) test for seedling growth enhancing effects after inoculation with *B. bassiana* or *P. lilacinum* as a seed treatment, and (2) evaluate the performance (i.e., fecundity) and behavior

(i.e., positioning) of sugarcane aphids on sorghum plants treated with *B. bassiana* or *P. lilacinum*.

Materials and Methods

Endophytic Fungal Strains

B. bassiana (Balsamo) (Vuillemin), commercially available as BotaniGard® (BioWorks Inc, Victor, NY), was mass cultured on Sabouraud dextrose yeast agar (SDAY) medium containing 8 g mycological peptone, 32 g dextrose, and12 g agar. Plates were incubated in a dark environmental chamber at 25^0C. *P. lilacinum* was isolated from cotton in a fungal community survey by Ek-Ramos *et al.,* (2013). Conidia of *B. bassiana* and *P. lilacinum* were separately harvested by adding 10 ml sterile water to each petri dish and scraping the surface of the medium with a sterile scapel into 15 ml conical falcon tubes. The resulting conidia suspension was mixed on a vortex and filtered to remove mycelia through sterile cheesecloth into a sterile beaker to obtain a stock solution. The conidial suspension of each fungus was then adjusted with sterile water to a final concentration of 1×10^7 ml^{-1} using a haemocytometer.

Sorghum Plants and Sugarcane Aphid

Sorghum cultivar ATx645/RTx437 was obtained from Dr. Bill Rooney (Texas A&M University, Department of Soil and Crop Sciences). Sugarcane aphids were obtained from a laboratory-maintained colony originally collected from sorghum fields in Corpus Christi, Texas. The colony was maintained on sorghum plants in an environmental chamber at 25^0C, 70% RH, and a 12:12 hr (L:D) photoperiod.

Seed Treatment with B. bassiana and P. lilacinum

Sorghum seeds were surface sterilized by immersion in 0.5% sodium hypochlorite (NaOCl) for 2 min and then in 75% ethanol for 2 min. The seeds were then rinsed three times in sterile water. Sterilized seeds were allowed to dry on sterile filter paper for 30 min. About 50 g of sterilized seeds were immersed in 20 ml of conidial suspension of each isolate, and another 50 g immersed in 20 ml water (as control) for 15 min. Seeds were allowed to dry for 30 min. The exact amount of fungal conidia attached to the seeds was not determined in this study. Fungal isolate preparation, seed sterilization, and seed inoculation were all performed under a laminar flow cabinet to ensure a sterile workspace.

Plastic cell pots (5.5 cm diameter and 8 cm depth) were filled with unsterilized Metro mix 900 soil (Sungro Horticulture, Agawam, MA) containing 40-50% composted pine bark, peat moss, vermiculite, perlite and dolomitic limestone. Four treated seeds were planted per pot and grown in an environmental growth chamber at 27^0C, 90% RH, in a 12:12 hr (L:D) photoperiod. Pots were placed in a completely randomized design and watered as needed. For each treatment (*B. bassiana*, *P. lilacinum* and control), one-week old plants intended for aphid performance assays were transplanted into cylindrical deep pots (6 cm diameter, 25 cm depth) and maintained under similar growth chamber conditions.

Assessment of Plant Height and Biomass

One-week old plants intended for assessing plant growth were divided into two groups to separately quantify growth at 14 and 30 days after planting. The plants were transplanted into 10 cm x 15 cm pots filled with unsterilized soil and maintained at growth chamber conditions (as above). Two-week old plants designated for assessing plant growth

30 days after planting were subsequently transferred to the greenhouse in a completely randomized design. For each group, plant height was measured from the soil surface to the tip of the uppermost leaf. After carefully removing soil from roots, fresh total biomass (g) was measured for each plant. Thirty-six individual plant replicates per treatment were used in this experiment.

Aphid Performance on Entire Plants 7 Days and 14 Days After Infestation

Two-week old plants grown in cylindrical deep pots and maintained in growth chamber conditions (25^0C, 70% RH, and a 12:12 hr (L:D) photoperiod) were used for this experiment. A single second or third instar aphid was transferred to individual plants per pot per treatment (i.e., *B. bassiana*, *P. lilacinum* and control). Aphids were carefully placed on the leaf collar of the second leaf at the start of the experiment. Cylindrical cages (6 cm diameter, 28 cm height) made from clear plastic and ventilated with no-see-um mesh (Eastex products, NJ) windows, were fitted tightly into each pot to cover plants. The top of each cage was plugged with polyurethane foam to prevent aphid escape. The effect of fungal treatment on the performance of sugarcane aphids was determined by: (1) counting the number of aphids on plants 7 days and 14 days after infestation, and (2) assessing whether fungal treatment influenced the position of aphids on plants 7 days and 14 days after infestation. Aphid position was categorized as whether the majority of aphids were located on the top of a plant (i.e., close to the tip of the upper leaves), or whether they were located on the bottom (i.e., the lower parts of the stem). Thirty replicates of individual infested plant per treatment were used in this experiment. Plants were watered as needed in equal amounts across treatment groups. The same experiment was repeated in a second trial.

Aphid Fecundity on "Top" Versus" Bottom" Plant Positions

To assess whether fungal treatment differentially influenced aphid fecundity with respect to aphid location on the plant, we conducted a no-choice experiment by restricting aphids to either the top or bottom of plants. Here, we refer to "bottom" as any region below the leaf collar of the second leaf to the soil surface, whereas "top" refers to any region above the leaf collar of the second leaf (Figure 16). "Top" and "bottom" regions of each plant were demarcated by a flat disc of polyurethane foam within a cylindrical cage sealed with cotton around the plant to prevent aphids from moving between regions. Each endophyte treatment group was divided into two subgroups with respect to whether aphids were introduced to the "top" or "bottom" of a plant. A single second or third instar aphid was placed on the respective region of each plant. Sixteen replicates per treatment (*B. bassiana*, *P. lilacinum* and control) per region (i.e., top vs. bottom) were used in this experiment.

Statistical Analysis

Data on aphid fecundity were first subjected to a square root transformation to homogenize variances. Transformed data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk normality test using SPSS V. 22 (IBM Inc., NY). Maulchly's test of sphericity was used to test for differences between treatments and time (i.e., 7 and 14 days after aphid infestation). For the first and second trials of aphid fecundity assessed on entire plants, a repeated measures ANOVA with time as a repeated factor was used to test for differences in aphid numbers 7 days and 14 days after infestation. To determine whether treatment had an effect on the position of aphids on a plant, we used a Pearson chi square test. Repeated measures ANOVA was performed on aphid fecundity assessed on "top"

versus “bottom” plant regions. Plant height and biomass data (square root transformed) were subjected to a one-way ANOVA. A significance level of alpha = 0.05 was used for all statistical tests.

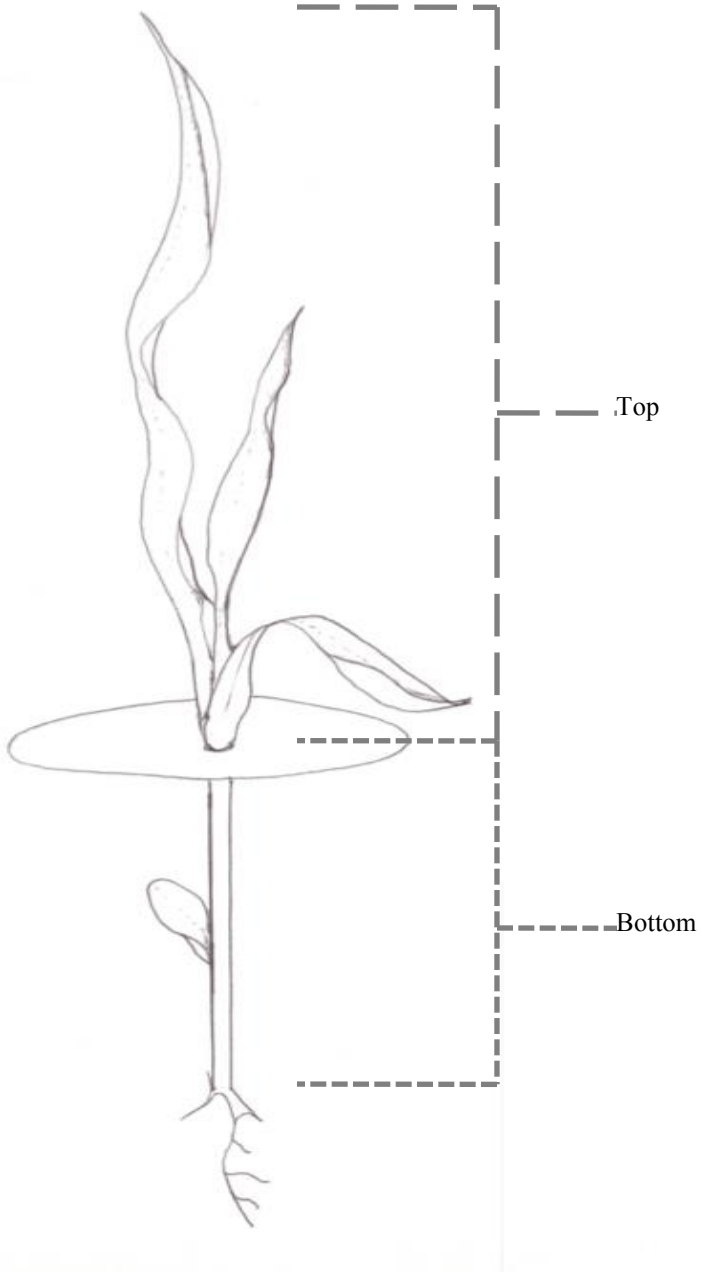

Figure 16. Locations on sorghum where aphids were introduced. “Bottom” refers to any region below the leaf collar of the second leaf to the soil surface, whereas “Top” refers to any region above the leaf collar of the second leaf. Photo by Toluwalase Okubote

Results

Assessment of Plant Height and Biomass

Endophyte treatment significantly increased plant height 14 days after planting ($F = 115.89$; $df = 2$; $P < 0.05$) but not 30 days after planting ($F = 1.03$; $df = 2$; $P = 0.36$). In contrast, endophyte treatment significantly increased total fresh biomass 30 days after planting ($F = 29.16$; $df = 2$; $P < 0.05$) but not 14 days after planting ($F = 4.64$; $df = 2$; $P > 0.01$) (Table 11).

Aphid Performance on Entire Plants 7 and 14 Days After Infestation

The effect of endophyte treatment on aphid fecundity differed significantly across trials ($F = 61.99$; $df = 1$; $P < 0.05$). Therefore, trials 1 and 2 were analyzed separately. In the first trial, aphid fecundity was significantly higher on plants treated with *P. lilacinum* than those treated with *B. bassiana* or the untreated controls ($F = 12.75$; $df = 2$; $P < 0.05$) and there was also a significant effect of time (i.e., between 7 days and 14 days after aphid infestation) ($F = 603.39$; $df = 1$; $P < 0.05$) (Figures 17 A and B). Additionally, there was a significant interaction between treatment and time ($F = 5.86$; $df = 2$; $P < 0.05$). Seven days after aphid infestation, aphids on plants treated with either *B. bassiana* or *P. lilacinum* segregated on upper parts of the plants (for consistency, we refer to this region as "top") while majority avoided the basal region of the plants (referred to as "bottom") (Table 12). In contrast, aphids were more commonly observed on the bottom region of control plants than on the top (Table 12). We did not observe such positional effect across all plants. Yet, overall, aphids seemed to segregate more on endophyte-treated plants than on control plants (Table 12). A Pearson's chi square test indicated that the differences in aphid position with respect to treatment were

significantly different 7 days after infestation ($\chi^2 = 23.62$; $P < 0.05$). Although aphids began spreading on the plant as their numbers increased, treatment effects on aphid position remained significant at the end of day 14 ($\chi^2 = 17.84$; $P < 0.05$).

In the second trial, aphid fecundity was significantly lower on plants treated with either *B. bassiana* or *P. lilacinum* than on control plants ($F = 6.99$; $df = 2$; $P < 0.05$). There was also a significant effect of time ($F = 333.22$; $df = 1$; $P < 0.05$) (Figures 18 A and B) but the interaction between treatment and time was not significant ($F = 2.34$; $df = 1$; $P = 0.11$). Endophyte treatment did not have an effect on aphid position 7 days after aphid infestation ($\chi^2 = 8.93$; $P = 0.18$). However, on day 14, there was significant effect of endophyte treatment on aphid position ($\chi^2 = 14.08$; $P = 0.03$). Here, more plants were infested at the bottom region compared to the top across all treatments, except in *B. bassiana*-treated plants where top infestation was more common than bottom (Table 12).

Aphid Performance on Restricted Plant Positions

Aphid numbers were higher on the top than on the bottom of the plant, but more aphids were found on the control than endophyte-treated plants (Figures 19 A and B). Aphid numbers were significantly different with respect to both treatment and aphid position at 7 days ($F = 12.49$; $df = 2$; $P < 0.05$ and $F = 17.28$; $df = 1$; $P < 0.05$ respectively) (Figure 19A) and 14 days after infestation ($F = 16.44$; $df = 2$; $P < 0.05$ and $F = 121.53$; $df = 1$; $P < 0.05$ respectively) (Figure 19B).

Table 11. Height and weight of sorghum treated with fungal endophytes. Estimates are based on 14-day old and 30-day old plants.

Treatment	Mean plant height (cm)	Total fresh biomass (g)
14 days		
Control	$17.5 \pm 0.39_A$	$0.49 \pm 0.07_A$
P. lilacinum	$20.4 \pm 0.29_B$	$0.62 \pm 0.02_A$
B. bassiana	$20.7 \pm 0.28_B$	$0.75 \pm 0.17_A$
30 days		
Control	$87.66 \pm 0.95_A$	$31. \pm 1.28_A$
P. lilacinum	$87.94 \pm 0.80_A$	$38.70 \pm 1.87_B$
B. bassiana	$86.16 \pm 0.84_A$	$41.25 \pm 1.71_B$

Different letters following mean plant height (±SE) and biomass (±SE) within the same column and number of days are statistically different at a significance level of P = 0.05

Table 12. Percentage of plants showing position effects on aphids. "Top" refers upper regions of plants whereas "bottom" refers to lower regions or plant. No segregation refers to plants on which aphids showed to shift towards a specific region of the plant.

	Treatment	Top (%)	Bottom (%)	No segregation (%)
Trial 1				
	Control	12	53	34
	P. lilacinum	61	17	23
	B. bassiana	47	33	19
Trial 2				
	Control	18	31	50
	P. lilacinum	27	42	30
	B. bassiana	21	15	61

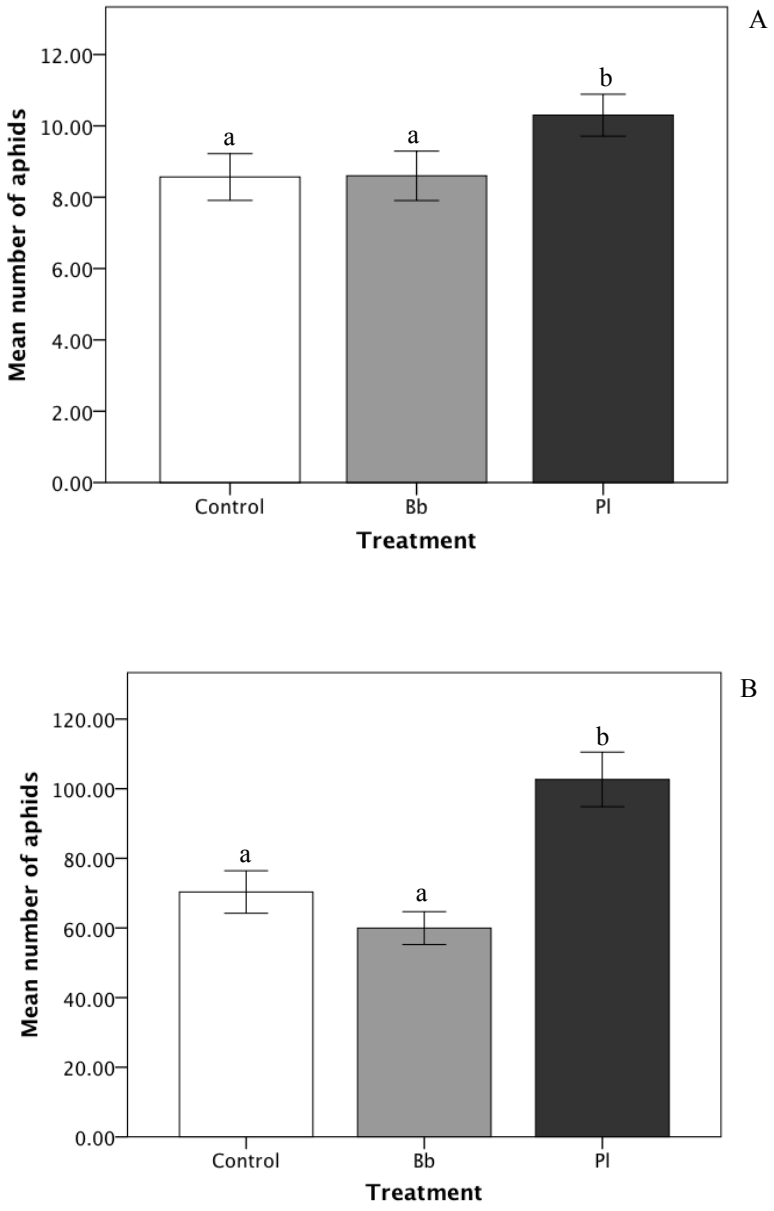

Figure 17. Mean (±1SE) number of aphids recorded on treated plants in trial one. Aphids were recorded on plants treated with *B. bassiana*, *P. lilacinum* and water (control) 7 days after aphid infestation (A) and 14 days after aphid infestation (B).

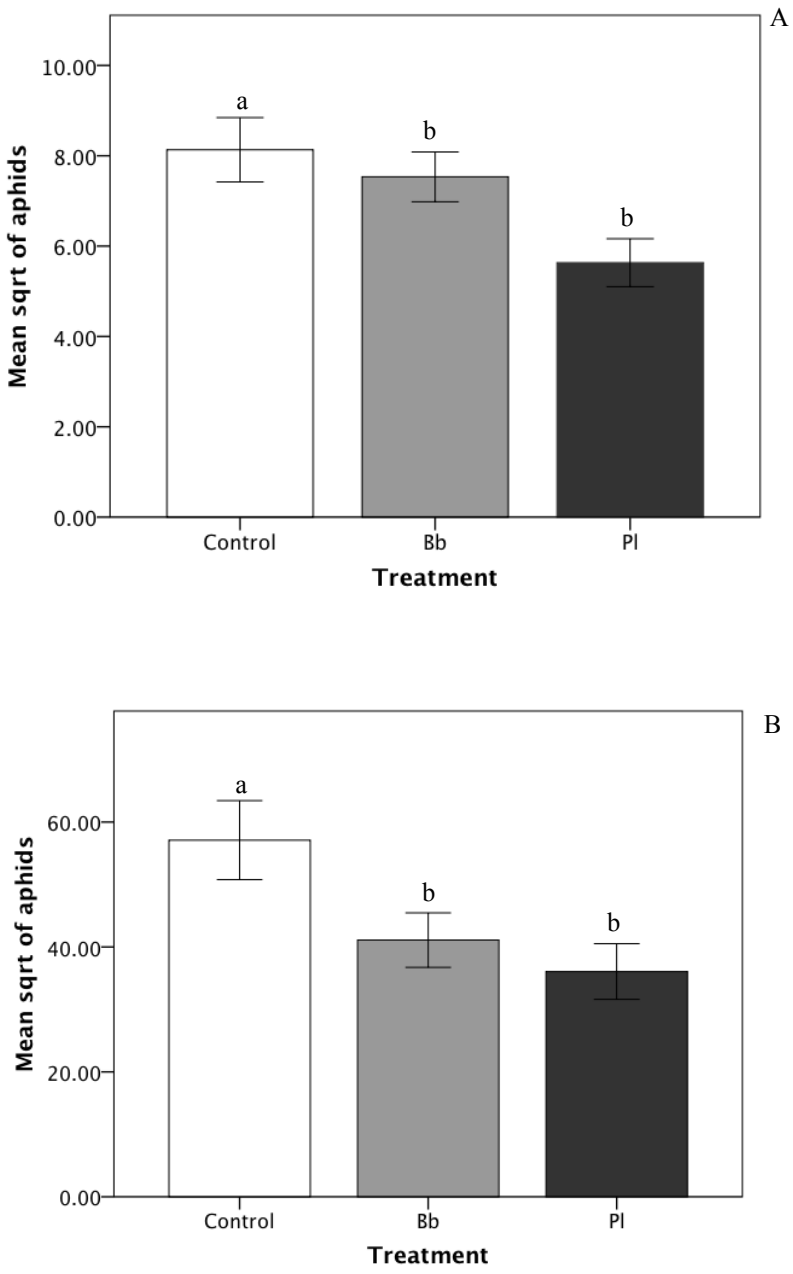

Figure 18. Mean (±1SE) number of aphids recorded on treated plants in trial two. Aphids were recorded on plants treated with *B. bassiana, P. lilacinum* and water (control) 7 days after aphid infestation (A) and 14 days after aphid infestation (B).

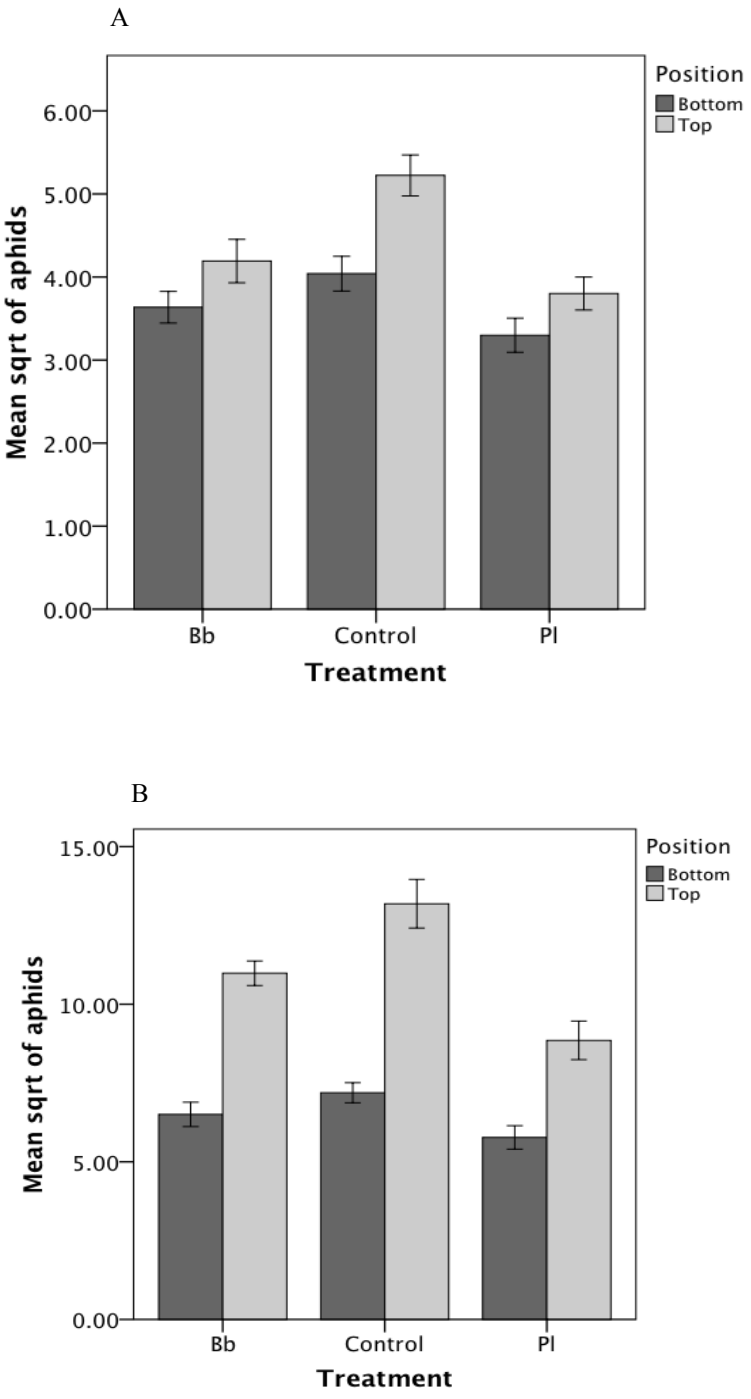

Figure 19. Mean (±1 SE) square root of aphids on treated plants by location. Aphids were recorded on plants treated with *B. bassiana*, *P. lilacinum* and water (control) 7 days after infestation (A) and 14 days after infestation (B).

Discussion

In this study I tested whether the performance of sorghum and the fecundity of sugarcane aphids feeding on sorghum would be affected by treating the plant with either *B. bassiana* or *P. lilacinum*. I also tested whether the relative position of sugarcane aphids on treated plants would differentially affect aphid fecundity. Although I did not directly quantify endophytic colonization in all of the plants used in these experiments, fungal endophyte metacommunity sequencing analysis conducted as part of another study indicates that sorghum can be colonized by both *P. lilacinum* and *B. bassiana* using the same seed treatment protocol used here (G.A. Sword, unpublished data). Previous studies have already demonstrated endophytic colonization of sorghum by *B. bassiana* (Mantzoukas *et al.*, 2015; Reddy *et al.*, 2009; Tefera & Vidal 2009). However, I cannot strictly rule out the possibility of soil-mediated effects either in addition to or instead of endophyte-mediated effects on the plant and insect responses we observed.

This study confirms that treatment with *B. bassiana* can improve sorghum and provides the first evidence for a plant growth enhancing effect of *P. lilacinum*. After soaking sorghum seeds in spore suspensions of *B. bassiana* and *P. lilacinum*, there was a significant increase in plant height at 14 days after planting. However, no such increase was detected when plants were older (i.e., 30 days after planting). Total fresh biomass of sorghum, on the other hand, was enhanced by endophyte treatment at both 14 days and 30 days after planting. I did not observe an increase in height in older plants possibly because of the change in environmental conditions when 14-day old plants were moved from a controlled environmental growth chamber to the greenhouse.

Direct and indirect effects of endophytic *B. bassiana* on insect herbivores have been reported (Akello *et al.,* 2008; Devi *et al.,* 2001; Gurulingappa *et al.,* 2011; Imoulan *et al.,* 2011; Lopez *et al.,* 2014; Reddy *et al.,* 2009; Wakil *et al.,* 2012). However, studies testing their potential to promote plant growth are relatively rare. Previous studies have attempted to test for plant growth-promoting effects of *B. bassiana* in sorghum (Reddy *et al.,* 2009; Tefera & Vidal 2009). Results from these studies were variable, from no effect on plant growth to increased growth. The present study showed that treatment of sorghum with *B. bassiana* can have plant growth-promoting effects (i.e., increased plant height and fresh biomass). Variation in the effect of *B. bassiana* on sorghum could be explained by differences in sorghum varieties and *B. bassiana* strains, which is not an uncommon outcome in plant-microbe interactions (Ball *et al.,* 2011; Furnkranz *et al.,* 2009; Gundel *et al.,* 2012; Qawasmeh *et al.,* 2012). Mutualistic interactions between plants and fungal endophytes are known to enhance plant growth (Vega *et al.* 2009) yet, much of the evidence showing that fungal endophytes enhance plant-growth comes from root endophytes such as *Metarrhizium anisopliae* (Berg 2009; Diene *et al.,* 2010; Elena *et al.,* 2001; Kabaluk & Ericsson 2007; Varma *et al.,* 1999). The ability of fungal endophytes to improve plant growth may not only be important to plant nutrition but may also protect plants from adverse environmental conditions and pest pressure (Johnson *et al.,* 2014; Singh *et al.,* 2004). In sorghum, for example, enhanced plant vigor as a result of an increase in plant height has been linked to reduce susceptibility to sugarcane aphid injury (Raman *et al.,* 2012).

Endophyte treatment had variable effects on aphid behavior and performance across our two replicate experiments (i.e., trials 1 and 2). In the first replicate, significantly more aphids were observed at the bottom than at the top of control plants relative to plants

inoculated with either *P. lilacinum* or *B. bassiana*. Although the number of aphids on *B. bassiana*-treated plants did not differ from those on control plants, three times more aphids were observed on plants treated with *P. lilacinum* by day 14. This finding contrasts with a previous report in cotton that showed that similarly treating cotton seeds with *P. lilacinum* negatively affected the fecundity of cotton aphids infesting the plants (Lopez *et al.,* 2014). However, in the second trial, both *B. bassiana* and *P. lilacinum* negatively impacted aphid fecundity, consistent with previous reports of negative effects of these fungi as endophytes on insect herbivores (Devi *et al.,* 2001; Gurulingappa *et al.,* 2010; Lopez *et al.,* 2014; Reddy *et al.,* 2009; Wakil *et al.,* 2012).

The variation in negative effects of *B. bassiana* and *P. lilacinum* on aphid fecundity that we observed may have been the result of positional effects of aphids on treated sorghum. Fungal endophytes associated with grasses (e.g., tall fescue) produce alkaloids that can either be toxic or deterrent to insect herbivores (Raman *et al.,* 2012). The distribution and concentration of these alkaloids are vital determinants of insect responses to plants (Raman *et al.,* 2012). Maximum concentration of fungal mycelia occurs at the stems and leaf sheaths whereas their concentrations in leaf blades are relatively low (Mathias *et al.,* 1990). The concentrations of their respective alkaloids are expected to follow a similar distribution (Raman *et al.,* 2012). Mathias *et al.,* (1990) reported an adverse effect of the relative position on chinch bug (*Blissus leucopterus*) performance on ryegrass (*Lolium perenne*). In their study, chinch bugs feeding on leaf sheaths of ryegrass treated with *Acremonium lolii* had significantly lower performance compared to those feeding on leaf blades of treated plants or any part of untreated plants. The authors attribute the low performance of chinch bugs on leaf sheaths to deterrence by higher concentrations of alkaloids produced by *A. lolii*. If this is true

for sorghum treated with *B. bassiana* or *P. lilacinum*, then endophyte-produced alkaloids could potentially be acting as feeding deterrents to sugarcane aphids. Additionally, aphids could be using the top region of treated plants as "enemy free" space where negative effects of endophyte treatment could be low. However, whether or not *B. bassiana* and *P. lilacinum* are directly responsible for such responses in sugarcane aphids on sorghum by virtue of alkaloid production is yet to be determined.

The fungal endophyte, *Neotyphodium coenophialum*, in tall fescue is thought to increase the nutritional quality (e.g., phosphorus and nitrogen) of their host plants (Lyons *et al.,* 1990; Malinowski *et al.,* 2000). It is possible that the increased performance of sugarcane aphids on *P. lilacinum*-treated, relative to *B. bassiana* and control plants, could be a nutritional effect of treating sorghum with *P. lilacinum.* Another possibility is that compensatory feeding in response to endophyte-mediated nutritional changes in the plant may have allowed aphids to perform better in the presence of the endophyte. It remains to be tested whether treating sorghum with *P. lilacinum* can change the nutritional composition of the plant for insect herbivores.

In the second trial, aphids did not show a strong positional effect by endophyte treatment, and may have succumbed to the negative effects of the endophytes independent of position as evidenced by their reduced fecundity on endophyte-treated versus control plants (Figure 18). When aphid location on plants was controlled I found that aphid fecundity was consistently lower on the bottom of the plant regardless of endophyte treatment (Figure 19). However, fecundity was lower on endophyte-treated plants than in control plants. Lower fecundity of aphids on the bottom of control plants relative to the top was rather unexpected given that aphids seem to favor lower parts of sorghum (Brewer, M., pers. communication;

pers. observation). One possible explanation is that there could be a higher frequency of naturally occurring sorghum endophytes (with strong herbivore resistance) within the lower portion of the plant, which may have negatively influenced aphid fecundity. Alternatively, demarcations created by the polyurethane foams may have induced microclimatic changes (i.e., reduced light and/or temperature to the lower plant region), which could be unfavorable to aphid reproduction (Hunt & Newman 2005).

Altogether, my results contribute to understanding the effect of asymptomatic fungal endophytes on plant development and their influence on plant resistance to insect herbivory. Findings from this study may lead to more sustainable ways of increasing plant vigor while conferring resistance to insects. I confirmed that treatment with *B. bassiana* can improve sorghum seedling vigor and for the first time report similar effects associated with *P. lilacinum* treatment. I have also demonstrated that treatment with these endophytes can negatively affect the fecundity of sugarcane aphids, but performance was conditional on both the identity of the endophyte and the location of aphids on endophyte-treated plants. In the future I hope to quantify the relative presence of fungi in plant tissues, their secondary metabolites and/or volatiles to test whether they are directly involved in changes in aphid fecundity and behavior on sorghum.

CHAPTER VI

CONCLUSIONS

My dissertation used an interdisciplinary approach involving both evolutionary and applied ecology to understand host-plant use by insects and the role that microorganisms play in this interaction. Insects' associations with host-plants may generate selective forces that drive genetic divergence (or host-associated differentiation (HAD)) in insect populations. This idea has generated many studies in evolutionary biology. Yet, much of these studies involve insects that spend multiple generations on those plants (e.g., parthenogenetic insects) or insects with narrow host-plant ranges. When sexually reproducing polyphagous insects have been tested for HAD, only a handful of their host-plants were involved. This approach limits our ability to detect the frequency of occurrence of HAD in natural populations of insect herbivores. In my dissertation, I tested the frequency of HAD in a sexually reproducing highly polyphagous insect, cotton fleahopper (CFH). A previous study detected 1 incidence of HAD when 3 of the insect's host-plants were tested. My dissertation extended the number of plant species to 13 (including cotton) with the idea that HAD could be more common with increasing sampling size. Based on amplified fragment length polymorphisms (AFLP) markers of CFH I detected 1 incidence of HAD on one wild host-plant species: scurvy mallow. This finding was interesting, especially when one compares my finding to that of the well-studied pea aphid. Original studies using pea aphids from 3 host-plants detected found 2 distinct genotypes. A recent study extended the number of host-plant species to include multiple species (i.e., 19 plant species) and detected almost a 4-fold incidence of HAD. One interesting difference in the biology of pea aphids and CFH is their

mode of reproduction (i.e., parthenogenesis vs. sexual reproduction, respectively). Sexually reproducing insects have a higher likelihood of gene flow and recombination than parthenogenetic insects. Since both factors are important in decreasing the likelihood for HAD to occur, they may have influenced the low frequency of HAD detected in CFH.

For an economically important insect like CFH low incidence of HAD suggests that dispersal from wild hosts to cotton fields might be common and that wild hosts should be taken into consideration when managing CFH. In contrast, genetic distinctness of CFH on scurvy mallow indicates that populations on this plant may be contributing less to CFH build-up on cotton fields.

Although intraspecific variation encoded in an insect's genome is an important force driving HAD there is also evidence that bacterial symbionts within insects influence population divergence when insects are associated with different host-plant species. To date, this idea has only been tested in parthenogenetic Aphidoidea or Phylloxeridae insects. Apart from their ability to spend multiple generations on a single host and maintain HAD on their respective hosts, these parthenogenetic insects acquire bacterial symbionts from their mothers, increasing the likelihood for a strong correlation between HAD and their symbionts. I tested the same idea in the sexually reproducing insect, CFH, associated with horsemint and cotton. These two populations exhibit HAD but examination of their microbiome did not reveal any correlation between microbiome and HAD. Although we currently do not know the mode of symbiont transmission in CFH, the low incidence of population divergence and a potential for symbionts to be horizontally acquired from different host-populations, make it less likely for CFH to maintain a host-plant associated microbiome. Thus, findings from my study suggest that vertical transmission of bacterial symbionts rather than host-plant

association per se may better explain differences in bacterial communities among host-associated insect populations.

An interesting finding in this study was the detection of *Wolbachia* in CFH from one of my study locations, Weslaco, and its complete absence in any other location. This finding suggests that some components of the insect microbiome may vary significantly among different geographic regions. Interestingly, I found that when present at a specific location, horsemint and cotton-associated CFH harbor different strains for *Wolbachia*. The functional role of *Wolbachia* in CFH is currently unknown. Future work is needed to explore the effect of *Wolbachia* infection in CFH. Work from my dissertation provides the first record of the microbiome of CFH. While the roles of most of the bacteria associated with the CFH remain unknown, my study detected some important bacterial pathogens of cotton for which CFH could act as a vector.

In my dissertation I proposed the idea that the microbiome of CFH may influence its pest status. Although, some specific bacteria have been found to explain pest status in other hemipterans, overall bacterial microbiome composition did not correlate with the pest status of CFH. However, bacterial communities were structured by location. The geographic clustering detected in my study reflects a regional clustering of CFH genotypes was previously in another study. In the future, manipulative experiments should be carried out to test the direct and interactive effects of climatic conditions and/or CFH genotypes on microbiome composition of CFH. In whiteflies, for instance, certain geographic biotypes of the insect are pests on potatoes because they host different bacterial communities. It would be interesting to know whether any such interplay between geographic variation and CFH genotypes predict the insect's pest status.

Just as bacterial symbionts within insects improve insects' host-plant use including allowing insects to utilize novel host plants, many symbiotic fungi improve plant growth as well as plants' resistance to insect herbivores. I explored the beneficial role of fungal endophytes in improving sorghum development and provide resistance against sugarcane aphids. I found that by treating sorghum seeds with two fungal endophytes, *B. bassiana* and *P. lilacinum* plant height and fresh biomass was enhanced 7 days and 30 days after planting. Additionally I have provided the first evidence for a plant growth enhancing effect of *P. lilacinum*. *P. lilacinum* is widely used as a soil inoculant to control soil-borne plant pathogens such as root-knot nematodes. Findings from my study also suggest that, in addition to their entomopathogenic effects, *B. bassiana* may benefit plant physiology. Enhanced plant growth is not only important to plant nutrition but may also protect plants from adverse environmental conditions and insect pressure.

I also found that treatment with either endophytes has the potential to negatively affect the performance (i.e., fecundity and behavior) of sugarcane aphids on sorghum. Variation in the effect of endophytes on aphids seems to vary depending on the position of the aphids on sorghum plants. Future studies should explore the relative concentration of fungal mycelia across the sorghum plant (e.g., in stems versus leaf sheaths or at different heights). Fungal endophytes may produce secondary products such as alkaloids and volatiles that can either be toxic or deterrent to insect herbivores. Therefore it will be interesting to know not only the variation in mycelial concentration but also whether variation in the relative concentrations of these products exists across endophyte-treated plants. My study shows promise to sugarcane aphid control on sorghum using endophyte inoculations. This insect was recently detected on sorghum in east Texas and within a few years has spread to

sorghum fields throughout the south-central U.S. Thus, I have not only provided evidence that fungal endophytes improve the development of sorghum but I have also established the potential for fungal endophytes to be included in IPM programs against sugarcane aphids.

Results from my dissertation have provided information on the role of host-plants in mediating genetic variation of insects and how microbes can influence host-plant use by an insect pest. My study has also provided evidence for the potential for some of these microbes to be included in field crop improvement and pest management for a successful implementation of IPM programs.

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